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Study on Two Chitin-Binding Proteins From Vibrio Parahaemolyticus.

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STUDY ON TWO CHITIN-BINDING PROTEINS FROM *VIBRIO PARAHAEMOLYTICUS*

A Dissertation

Submitted to the Graduate Faculty of the
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in

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by
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Dedication

Without a doubt, no one deserves this dedication more than the person who has stood with me for all these years, sharing the frustrations of the narrow path.

To the mother of my children,
my beloved wife,

Tatjana

Acknowledgement

This small contribution has one visible author but many silent contributors. I especially wish to thank the members of my Committee, Drs. Sue Bartlett, Jesse Jaynes, Marion Socolofsky and Ezzat Younathan for their advice, support and friendship. Thanks go to my advisor Dr. Roger Laine, for always finding ways to help me keep my mind young and active. His contagious enthusiasm in research is greatly appreciated. The help of Dr. Betty C.-R. Zhu for being always available to discuss results and questions involving protein purification procedures is greatly appreciated.

The setting has been strange, the road full of surprises. I shall not forget the friends I made here.

It has been a long journey

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Abstract

Two chitin-binding proteins from *Vibrio parahaemolyticus* are secreted into the culture medium after the addition of chitin or oligomers of N-acetylglucosamine, but only to lower levels of expression with a few other saccharides. The first protein exhibited a very salt-resistant chitinase activity with activity maxima at pH 6 and 9. The enzyme is stable for 30 minutes at 40°C, and maximal activity is reached at 50°C. The chitinase hydrolyzes optimally chitin oligomer substrates with degree of polymerization of 4 and higher. The hydrolytic activity upon the chitotrimer is low; the dimer cannot be hydrolyzed by the enzyme. When incubated with chitotrimer, the protein exhibits transglycosylase activity. The chitinase from *V. parahaemolyticus* shows a strong sequence homology with chitinases from *Serratia marcescens* and *Bacillus circulans*.

The other protein purified from *Vibrio parahaemolyticus* culture supernatant fractions is a chitin-binding protein with an apparent molecular weight of 134,000. The protein was either purified by conventional ammonium sulfate precipitation followed by fractionation on a gel filtration column, or by affinity chromatography on regenerated chitin, followed by differential elution with increasing concentrations of guanidine hydrochloride. Samples of this protein eluted with guanidine hydrochloride retained their binding capacity after dialysis. The protein could

also be specifically eluted from chitin with a concentrated solution of chitin oligomers. The N-terminal amino acid sequence of the protein showed no strong homolgy to any known protein sequences in the *GeneBank* data bank, suggesting that it may be a novel carbohydrate-binding protein tentatively named *chitovibrin*. Chitovibrin exhibits similar chromatographic characteristics to chitinase, but polyclonal antibodies raised against chitinase do not cross-react with chitovibrin. The function of chitovibrin has yet to be determined.

Chapter 1

Introduction

1.1 Chitin in nature

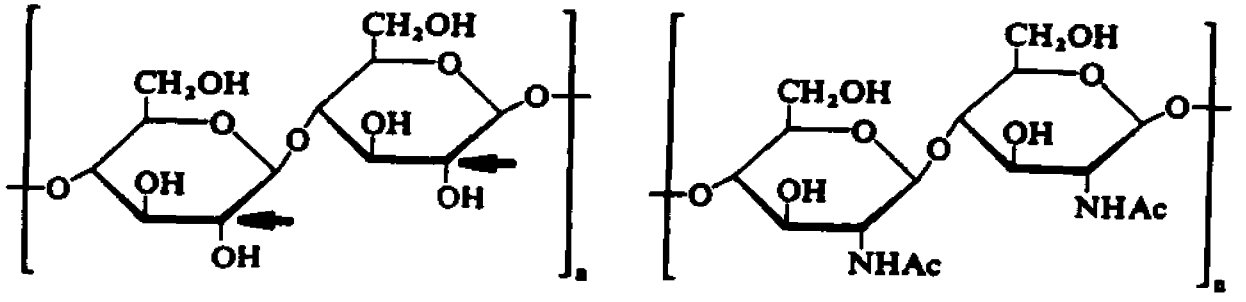
Except for cellulose, chitin is the most abundant renewable source of carbohydrates, a strong indication of its relevance in nature. Enormous quantities of chitin are synthesized and degraded each year. In the aquatic environment alone approximately 10^{11} metric tons are produced annually (Tracey, 1957). According to Foster and Webber (1960), chitin was first observed by Bracconot in 1811 in the residuals from fungal extractions. He named this alkali-resistant material "fungine". In 1823, Odier proposed the name chitin (from the Greek, tunic or covering) to the material he prepared from wings of May beetles. Later, the name chitin was used for the compound in plant and animal kingdoms. Chitin consists predominantly, if not entirely, of unbranched homopolymer chains of β1->4 linked N-acetyl glucosamines, with an average molecular weight of 10^6 Da for chitin purified from crabs (Brine and Austin, 1981), to higher than 1.8×10^6 Da for chitin from other crustaceans (Shimahara *et al.*, 1984). Chitin is the principal constituent of the exoskeleton and linings of internal body parts of insects (Shimahara and Takiguchi, 1988), as well as mollusks, shrimp, crayfish and crabs (No *et al.*, 1989). Chitin also is found as a component of nematodes and fungal cell walls, particularly the bud scars (Barrett-Bee and

Hamilton, 1984). In the cuticle of insects and crustaceans, chitin always occurs combined with other substances like calcium carbonate, calcium phosphate, pigments and proteins, forming a layered and rigid structure. The protein present may be extractable with hot water or may be covalently attached to the polysaccharide. Chitin sediments produced by molted copepod exoskeletons alone, have been estimated at several billion tons each year (Muzzarelli, 1973).

1.2 Chemical composition and structure of chitin

Chitin can be formally considered a derivative of cellulose in which the C-2 hydroxyl groups have been replaced by acetamido groups, as shown in *Figure* 1.1.

Fukamizo *et al.* (1985) showed that chitin prepared from tobacco hornworm cuticle or from crab contains trace levels of deacetylated residues which are released as dead-end products after enzymatic digestion. Three crystalline forms of chitin have been reported: α, β and γ. The main structural feature of these different forms is that chitin chains form parallel (for α-chitin) and antiparallel sheets (for β-chitin) linked by hydrogen bonds. The unit cell of α-chitin is similar to that of cellulose: it contains four glucopyranose rings linked by two pairs of (1->4)-ß-glucosidic bonds. There is extensive intermolecular hydrogen-bonding between carbonyl (C=O) and amino (N-H) groups, but no apparent bonding between C=O and O-H in the chitin crystals. The structure



CELLULOSE **CHITIN**

Figure 1.1 **Structures of cellulose and chitin.** The hydroxyl group on carbon 2 (arrows) in cellulose is substituted by an acetamido group in chitin.

of β-chitin is very similar but it apparently contains one water molecule per acetylaminoglucose unit. In γ-chitin, the chitin chains occur in groups of three, each group possibly formed by the folding of a single chain (two parallel, one antiparallel). Animal chitin regularly appears associated with protein, either covalently linked or held together by hydrogen bonding. The structure of chitin and that of proteins in β-sheet conformation appear to be very complementary so as to form interpenetrating lattices. Fungal chitin, on the other hand, appears to be associated mainly with a polyglucan linked β(1->3) and β(1->4). The only known source of pure chitin, i. e. polymer synthesized in the absence of protein or other macromolecules, occurs in certain diatoms which have spines of highly crystalline β-chitin (Blackwell and Weih, 1984).

1.3 Chemical degradation of chitin

The structural characteristics of chitin described above help explain the tenacious nature of the compound. Purified chitin is a white, amorphous solid that is insoluble in water, dilute acids, dilute and concentrated alkalies and all organic solvents. It can be dissolved readily in anhydrous formic acid, in hypochlorite solutions, and in concentrated mineral acids (Conrad, 1965).

Oligomers of N-acetyl glucosamine can be readily obtained by hydrolysis of purified, commercially available chitin with concentrated inorganic acids. The partial hydrolysis of chitin with

concentrated hydrochloric acid over a range of temperatures and acid concentrations has been reported by Rupley (1964). One of the disadvantages of the method described is the partial de-N-acetylation of the resulting oligomers. A more convenient way of preparing chitin-oligosaccharides was described by Bosso *et al.* in 1986, and it involved the hydrolysis of chitin with hydrogen fluoride. The yield was almost quantitative and the average degree of polymerization depended both on reaction time and temperature. No apparent de-N-acetylation in the products was observed.

A solvent system consisting of 5% or 8% lithium chloride in N,N'-dimethylacetamide has been used (Brine and Austin, 1981 and Bosso *et al.*, 1986), especially when only the solubilization of chitin is desired, without producing the extensive depolymerization obtained upon highly acidic conditions. Solubilization of the polysaccharide takes several days but is easy and requires no previous activation. A controlled ultrasonication of these solutions allows the production of chitin samples with narrow molecular weight distribution (Terbojevich, *et al.*, 1988).

1.4 Biodegradation of chitin

In recent years, the potential use of chitin in the food industry as well as a source of pharmaceuticals and animal and aquaculture feed has been pursued in view of the enormous

amounts of land-fill material arising from seafood processing plants. With an average chitin content of 15% of the dry weight of the chitinous waste products, the total estimates of annually accessible chitin amount to 150,000 metric tons globally (Knorr, 1984), 1/3 of which is dumped in landfills or hauled out to sea in the United States alone.

A key step in the processing of seafood waste is the bioconversion of the chitinous material by enzymes from selected microorganisms (Carroad and Tom, 1978). Process plants have been devised in order to recover and economically utilize the available chitin from seafood wastes (No *et al.*, 1989). The implementation of such plants would also lift some of the burden on the environment and allow a better use of the land-fill sites.

Chitinases, the enzymes which degrade chitin, are widely distributed in nature. They have been isolated from bacteria and fungi in the soil, from microorganisms, invertebrates and vertebrates in marine environments, as well as from insects, plants, and the sera of some animals. The complete, natural degradation of chitin is accomplished by the synergistic action of *chitinase* and *chitobiase*. Chitinase (EC-3.2.1.14) hydrolyzes chitin to produce N,N'-diacetyl chitobiose, which is further hydrolyzed to N-acetyl glucosamine (GlcNAc) by the action of chitobiase (EC-3.2.1.30).

Some oligomers of N-acetylglucosamine are known to have important biological activities which include immunostimulation and tumor-growth inhibition. $(GlcNAc)_6$ and $(GlcNAc)_7$ show marked antitumor acvtivity in Balb/c mice Sarcoma 180 solid tumors (Suzuki *et al.*, 1986). The synthesis of these chitin oligosaccharides by enzymatic hydrolysis has been shown to reduce low yield and high deacetylation obtained by chemical methods (Shimahara *et al.*, 1984). Hexa-N-acetylchitohexaose and hepta-N-acetylchitoheptaose were efficiently produced through the transglycosylation activities of chitinases from *Trichoderma reesei* and *Nocardia orientalis* (Usui *et al.*, 1987), and of lysozyme (Usui *et al.*, 1990). Many workers have reported on the purification of chitinases and chitobiases from bacteria including the species *Bacillus* (Cody, 1989), *Clostridium* (Pel *et al.*, 1990), *Streptomyces* (Berger and Reynolds, 1958; Hara *et al.* 1989, and Okazaki and Tagawa, 1991), *Vibrio* (Ohtakara *et al.*, 1979; Soto-Gil and Zyskind, 1984; Wortman *et al.* 1986; Jannatipour *et al.*, 1987), *Serratia* (Roberts and Cabib, 1982; Jones *et al.*, 1986; Fuchs *et al.*, 1986; Joshi *et al.*, 1988; Kless *et al.*, 1989) and *Aeromonas* (Roffey and Pemberton, 1990). Chitinolytic enzymes also have been detected in fungi (St. Leger *et al.*, 1986, Pedraza-Reyes and Lopez-Romero 1989 and 1991; Vasseur *et al.*, 1990), yeast (Correa *et al.*, 1982; Barrett-Bee and Hamilton, 1984), as well as in crustaceans (Funke and Spindler, 1989; Lynn, 1990) and insects (Koga *et al.*, 1982 and 1983; Chen *et al.*, 1982; Fukamizo and Kramer, 1985). Huber *et al.*, recently (1991) reported that malaria parasites gain

access to the peritrophic membrane of the midgut of the insect vector by the action of a chitinolytic activity present in the parasite. In higher plants, chitinase has been purified from seeds (Molano *et al.*, 1979; Leah *et al.*, 1991), but more commonly, it has been demonstrated in other plant tissues, elicited as a pathogenic-related response upon parasitical attack (Boller, 1986; Kombrink *et al.*, 1988; Vœgeli-Lange *et al.*, 1988). Ethylene, a plant hormone, induces the synthesis of chitinase in bean leaves (Boller *et al.*, 1983).

1.4.1 Chitin hydrolases

Enzymes responsible for the hydrolysis of chitin are endo-chitinases (Tsukamoto *et al.*, 1984) and exo-chitinases (Takiguchi and Shimahara, 1988), in addition to chitobiases (Monreal and Reese, 1968), which, in some cases, can hydrolyze the di-N-acetylchitobiose substrate from the reducing end (Kuranda and Aronson, 1986). Lysozymes exhibit some chitin-hydrolyzing activity (Pahud *et al.*, 1983).

1.4.2 Chitin hydrolases in bacteria

A large number of different chitinase-producing bacteria are found in environments rich in chitin originating from crustaceans, insects or fungi. In general, such microorganisms are chitinoclastic, i.e. they synthesize both chitinase and chitobiase (Monreal and

Reese, 1968; Charpentier and Percheron, 1983; Yabuki *et al.*, 1986). The chitinase is usually an endo-chitinase, hydrolyzing glycosidic linkages of chitin at random positions, and generating di-N-acetylchitobiose. Strain E-383a from *Vibrio anguillarum* has been reported to produce only one chitin hydrolase, an exo-chitinase (Takiguchi and Shimahara, 1988). Yabuki *et al.* (1986) reported the glycoprotein nature of a chitinase and a chitobiase from *Aeromonas hydrophila* subspecies *anaerogenes* A52. Chitobiase from *Vibrio harveyi* was suggested to be a lipoprotein (Soto-Gil and Zyskind, 1989). Cloning of the chitinase gene from *Serratia marcescens* revealed the existence of five different chitinolytic proteins with masses ranging from 21 kDa to 57 kDa (Fuchs *et al.*, 1986). Bacterial chitinases or chitobiases have been cloned into *Escherichia coli* from *Serratia* (Jones *et al.*, 1986; Joshi *et al.*, 1988), *Aeromonas* (Chen *et al.*, 1991), *Vibrio* (Wortman *et al.*, 1986) and *Streptomyces* (Robbins *et al.*, 1988). Cloning into *E. coli* of a *Bacillus* chitinase revealed sequence homology to type III homology units of fibronectin (Watanabe *et al.*, 1990). Cloned chitinases are generally produced constitutively (Jannatipour *et al.*, 1987; Kless *et al.*, 1989), although Chen *et al.* (1991) observed that the expression of a 85 kDa chitinase from *Aeromonas hydrophila* was inducible by chitin and strongly repressed by glucose, when cloned into *E. coli*. According to Wortman *et al.* (1986), expression of native chitinases can be induced by chitosan, chitin, N-acetylglucosamine, or glucosamine.

The effects of chitinase on fungal cell wall degradation (Oranusi and Trinci, 1985; Beyer and Diekman, 1985) has been examined. Insect control mediated by the chitinase activity from *Serratia marcescens*, a known insect pathogen, has been reported by Lysenko (1974). Sakuda *et al.* (1987) reported a different mode by which bacteria attack insects: a *Streptomyces* species produces an insect growth regulator, termed allosamidin, which inhibits insect chitinase, thus interfering with ecdysis.

Generally, bacterial chitinases display activity at a wide range of pH and temperature (Ohtakara *et al.*, 1979; Roberts and Cabib, 1982; Yabuki *et al.*, 1986; Hara *et al.*, 1989). The enzymes reported exhibit molecular weights ranging from 27,000 (Fuchs *et al.*, 1986) to 110,000 (Yabuki *et al.*, 1986), and isoelectric pH from 3.1 to 8.8 (Okazaki and Tagawa, 1991). Chitinases hydrolyze partially N-acetylated chitosan with different degrees of specificity toward the N-acetyl-ß-D-glucosaminidic bonds (Mitsutomi *et al.*, 1990; Ohtakara *et al.*, 1990).

1.4.3 Chitin hydrolases in eukaryotes

A wide variety of eukaryotic species exhibit chitinolytic activities. These enzymes are involved either in developmental, invasive, nutritional or defensive processes of the organisms. The occurrence of chitinases in insects and their involvement and relevance in cuticle degradation during molting is well documented

(Chen *et al.*, 1982, Koga *et al.*, 1982 and Spindler-Barth *et al.*, 1986). Mommsen (1980) reported the existence of a chitinase/ß-N-acetylglucosaminidase enzyme system in the digestive fluid from a hunting spider. Lynn (1990) reported a similar system in the gastric juices from lobsters. Two of the lobster chitinases as well as a chitinase purified from brine shrimp (Funke and Spindler, 1989) and the chitin hydrolase from *Manduca sexta* (Koga *et al.*, 1983) are glycoproteins. Fungal enzymes have been implied in autolysis during apical growth, wall softening during hyphal branching and during germination (Reyes *et al.*, 1989 and Pedraza-Reyes and Lopez-Romero, 1989). The fungus *Aphanocladium album*, hyperparasite of wheat rust, secretes a chitinase which is an important element in the attack and lysis of the fungal host (Srivastava *et al.*, 1985). Smith and Grula (1983) and St. Leger *et al.* (1986) confirmed the presence of inducible chitinases during penetration of the insect cuticle by entomopathogenic fungi. In studies reported by Lundblad *et al.* (1974 and 1979), chitinase activity was found in the serum of various animals. In mammals, the glycosidase responsible for splitting the GlcNAc-D-(ß1->4)GlcNAc moiety in Asn-linked glycoproteins is a chitobiase, found in the lysosomes, which hydrolyzes the substrate from the reducing end (Kuranda and Aronson, 1986, and Aronson *et al.*, 1989).

Although plants express chitinase, the enzyme has no apparent function in their developement or growth. Chitin is

uncommon in plants but, together with ß-1,3-polyglucans, is the main component of the fungal cell walls. Plant chitinases have been demonstrated to be potent fungal growth inhibitors (Schlumbaum *et al.*, 1986). The presence of chitinase in plant seeds is one of the mechanisms that plants use for protection against pathogenic attack (Molano *et al.*, 1979; Leah *et al.*, 1991; Zhu and Lamb, 1991). Chitinases and glucanases are induced in other plant tissues following interaction with pathogens or upon treatment with chemicals (Abeles *et al.*, 1971; Nasser *et al.*, 1988; Vœgeli *et al.*, 1988; Vœgeli-Lange *et al.*, 1988; Kombrink *et al.*, 1988; Sauter and Hager, 1989). The plant hormone ethylene is an inducer of chitinase in melon leaves (Roby *et al.*, 1986) and of chitinase and ß-1,3-glucanase in bean leaves (Mauch and Staehelin, 1989).

The deduced amino acid sequences for the basic and acidic chitinases from *Arabidopsis thaliana* show 73% amino acid similarity to the basic chitinase from tobacco and 60% similarity to the acidic chitinase from cucumber, respectively (Samac *et al.*, 1990). RCH10, a rice chitinase gene, has 63% identity at the nucleotide level and 75% identity at the amino acid level with chitinase genes genes from bean, potato and tobacco (Zhu and Lamb, 1991). The amino-terminal sequence of the bean-leaf chitinase has revealed similarities to the sugar-binding domain of wheat germ agglutinin (Lucas *et al.*, 1985).

Plants commonly synthesize more than one species of chitinase. Yam (Tsukamoto *et al.*, 1984), maize (Nasser *et al.*, 1988), and barley seed (Leah *et al.*, 1991) contain multiple forms of the enzyme. Shinshi *et al.* (1990) suggested that the structures of the different chitinase genes in tobacco arose through transposition of sequences encoding a domain rich in cysteine residues. Nine different chitinase species were apparent in germinating cells of the fungus *Mucor rouxii* (Pedraza-Reyes and Lopez-Romero, 1991).

1.5 Carbohydrate-binding proteins and lectins

Carbohydrates are the most abundant organic compounds in the biosphere. In living cells their importance is central in the storage and transfer of energy as well as for mechanical work and in chemical reactions. In plants, they also play a key role in maintaining the structure of the cellular entity. Cells can express a vast number of intracellular proteins and enzymes that bind carbohydrates that are involved in many important biological functions. For survival purposes, living organisms also have the capacity to recognize the difference between self and non-self. When coming in contact with extracellular compounds or with other organisms, some of the proteins involved in the recognition are sugar-specific and able to agglutinate cells, or mediate cellular adhesion (Sequeira, 1978; Mirelman and Ofek, 1986). They are called lectins and were first discovered in plant extracts. Lectins

are defined as carbohydrate-binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates (Pistole, 1981). Lectins have been isolated from microorganisms (Mirelman and Ofek, 1986), invertebrates and vertebrates (Yeaton, 1981; Lis and Sharon, 1986), and plants (Lis and Sharon, 1973). Classification of lectins is based on the fact that their binding to carbohydrates or glycoproteins can be specifically inhibited by monosaccharides. In recent years, the widespread occurrence of lectins in plants, animals and microorganisms has been firmly established. Generally, lectins are glycosylated and composed of homo- or heterodimers with one binding site per subunit (Lis and Sharon, 1986). An unusual lectin from stinging nettle, that exhibits binding specificity towards chitin reportedly consists of one polypeptide chain (Peumans *et al.*, 1984; Shibuya *et al.*, 1986).

In microorganisms, extracellular and membrane-bound proteins are involved in the recognition and eventual incorporation of sugars into the cell. This assimilation event is mediated by a variety of enzymes and transport proteins which may be constitutively expressed or may be induced in the cell by specific effector molecules.

1.5.1 Chitin-binding proteins in eukaryotes

Many organisms produce carbohydrate-binding proteins such as lectins. They were first discovered and are more abundant in plants. Lectins that bind specifically to N-acetylglucosamine have been observed in *Solanum tuberosum* (potato) (Matsumoto *et al.*, 1983), *Datura stramonium* (jimson weed) (Crowley and Goldstein, 1981), *Luffa acutangula* (Ridge gourd) (Anantharam *et al.*, 1986), *Oryza sativa* (rice) (Poola, 1989), *Laburnum alpinum* (Konami *et al.*, 1983), *Urtica dioica* (stinging nettle) (Shibuya *et al.*, 1986), *Triticum vulgare* (wheat) (Lis and Sharon, 1973) and *Lycopersicon esculentum* (tomato) (Nachbar and Oppenheim, 1982). A chitin-binding haemagglutinin with a molecular weight of 86,000 is secreted by the fungus *Conidiobolus lamprauges* (Ishikawa *et al.*, 1981). A haemagglutinin activity from *Entamoeba histolytica* is membrane-associated (Mirelman and Ofek, 1986). Some lectins from invertebrates like sponges, gastropods and arthropods, bind chitin-substrates (Yeaton, 1981).

1.5.2 Chitin-binding proteins in bacteria

Bacteria exhibit cell-agglutination activity, which can be inhibited by simple sugars. In general such binding activities exist as fimbriae or pili, which are filamentous appendages expressed by many Gram-negative bacteria when grown under suitable conditions, and which do not function in motility. Fimbriae consist of arrays of identical protein subunits, known as pilin, of

approximately 170 amino acids. Different types of fimbriae showing distinct sugar specificity are involved in attachment to animal cells. The most common are mannose-specific (type 1), and galactose-specific (type P) fimbriae. Very few non-catalytic chitin-binding proteins have been isolated from microorganisms. *Chlamydia trachomatis*, *Bordetella bronchiseptica*, *Pasteurella multocida* and *Streptococcus salivarius* have been shown to express specificity of binding to N-acetylglucosamine. The association of these activities with a particular cellular structure has not been established (Mirelman and Ofek, 1986).

Yu *et al.* (1987) reported on the existence of an adhesion/deadhesion apparatus in *Vibrio furnissii* where a proposed simple lectin, with a broad sugar specificity, mediates the binding of the bacteria to glycosides of N-acetylglucosamine, glucose or mannose linked to Agarose beads. No proteins exhibiting these binding activities have yet been characterized or isolated from this system.

Some carbohydrate-binding activities in bacteria have been observed which do not appear to be pilin-associated. In the non-fimbriated *E. coli* 2699, Eshdat *et al.* (1981) isolated membrane vesicles showing stronger agglutinating activity than that of fimbriae from the organisms grown under more favourable conditions. No intact or fragmented fimbriae were detected in the membrane vesicles. These observations led Eshdat *et al.* to suggest

the existence in the preparations of protein subunits different than pilin that bind carbohydrates.

Chapter 2

Vibrio parahaemolyticus

2.1 Natural occurrence of *Vibrio* organisms

Members of the *Vibrio* genus are Gram-negative, rod-shaped bacteria that can be isolated from freshwater, estuarine and seawater environments. These organisms, generally found associated with chitinous plankton, shellfish, crustaceans and copepods, have also been isolated from the digestive tract of man and other warm-blooded animals (West and Colwell, 1984). *Vibrio cholera* is suggested to be a native species of brackish waters and estuarine bacterial communities, since it is widespread in natural marine environments in the absence of cholera and gastroenteritis cases (Huq *et al.*, 1984). *Vibrio parahaemolyticus* is a mildly halophilic organism that utilizes glucose fermentatively and grows best in the presence of 2 to 6% sodium chloride. *V. parahaemolyticus* is most prevalent in estuarine and inshore coastal waters from which shellfish and seafood are harvested. Association of *V. parahaemolyticus* with marine fauna was reported to be essential for the survival of the organism and the continuation of its the annual cycle in Chesapeake Bay (Kaneko and Colwell, 1975). Vibrios are capable of both fermentative and respiratory metabolism and can survive for prolonged incubation periods under strict anaerobic conditions (Youngren-Grimes *et al.*, 1988).

2.2 Ecological impact of *Vibrio* species

The relevance of the *Vibrio* genus on public health has been clearly established in the last 20 years. Mainly due to the virulence of the disease and also for historical reasons, most of the research on human pathogenic vibrios has been centered on *Vibrio cholera*. The recent discoveries of other *Vibrio* species involved in highly severe infections has led to a reassessment of the ecological impact of the *Vibrio* genus on humans.

Although at least 11 of the species of the genus *Vibrio* are known to be pathogenic to man, *V. cholera*, *V. parahaemolyticus* and *V. vulnificus* are of primary medical importance for the high health risks involved upon infection. Other organisms implicated as oportunistic pathogens causing gastroenteritis or wound infections in persons with poor or compromised health are *V. alginolyticus*, *V. damsela*, *V. fluvialis*, *V. furnissii*, *V. hollisae*, *V. mimicus*, *V. metschnikovii and V. cincinnatiencis*. It has been reported that 50% of the cases of diarrhea in the 0-4 year age group in Bangladesh is caused by *V. fluvialis* (Myatt and Davis, 1989). By far the most pathogenic of these species is *V. cholera*, which expresses a multi-subunit extracellular enterotoxin that stimulates secretion of isotonic fluid by small intestine enterocytes. Upon ingestion of the organism, virulence of a particular strain depends not only on the levels of enterotoxin produced, but also on the susceptibility of host defenses, of which an important factor is the acidity of the gastric acid (West, 1989).

The close association of *V. parahaemolyticus* with marine organisms explains the high incidence of gastroenteritis and extraintestinal infections in coastal areas, especially during warmer months, when the organism is most prevalent. The symptoms associated upon infection with *V. parahaemolyticus* include mild diarrhoea, abdominal cramps, nausea, vomiting and some fever. Extraintestinal infections are generally not severe and occur generally after exposure of skin lesions to saline aquatic environment or after handling seafood contaminated with *V. parahaemolyticus*.

Some vibrios are pathogenic to marine vertebrates and invertebrates, which makes them economically important. The ecological relationship of vibrios with aquatic fauna has been well documented. It has been suggested that the association of these organisms with the chitin component of plankton, shellfish and fish enables them to prolong their existence and maintain their numbers (Dastidar and Narayanaswami, 1968; Kaneko and Colwell, 1973 and 1975; Nalin *et al.*, 1979; Karunasagar *et al.*, 1986; West, 1989). The concentration of pathogenic vibrios in natural aquatic environments is very dependent on the water temperature. Most pathogenic vibrios disappear from the water column when the temperature is lower than 10°C and higher than 30°C (DePaola *et al.*, 1990). Even outside of this temperature range, pathogenic vibrios can be isolated from sediments and shellfish. As most

species reappear when conditions are more favorable, it is likely that, at some stage, all pathogenic vibrios become associated with chitinous parts of plankton and shellfish. The attachment of *Vibrio cholera* to copepods may explain the epidemiology of cholera in certain coastal areas, where the incidence of the disease is seasonal and endemic (Huq *et al.*, 1984). *V. cholera* was observed to attach specifically to the surfaces of live copepods, which was suggested to be relevant in the ecology of the species, as well as in the epidemiology of cholera (Huq *et al.*, 1983). Adherence to chitin may be a significant factor influencing the high survival rate of colonies in the acidic environment of the gastric tract of crabs (Nalin *et al.*, 1979). After observing that some of the vibrio species eliminated others in mixed cultures, Dastidar and Narayanaswami (1968) suggested that chitinous debris was the common environmental source where vibrios could multiply, provided they could synthesize chitinase.

2.3 Extracellular proteins of *Vibrio* species

The proteins described in this study are expressed by *Vibrio parahaemolyticus* extracellularly. The information available on proteins secreted by *Vibrio* organisms indicates the proteins described in this report have not yet been characterized.

Although Gram-negative bacteria generally do not secrete large amounts of proteins into the growth medium as Gram-positive bacteria do, members of the family *Vibrionaceae* are

known to produce different extracellular proteins including proteases, amylases, DNases, fibrinogen hydrolases, lipases and haemolysins (Myatt and Davis, 1989).

Examination of 27 *Vibrio* strains from species *V. cholera*, *V. furnissii*, *V. mimicus* and *V. fluvialis*, revealed the expression of chitinase in all of them. According to Huq *et al.* (1983), all pathogenic vibrios synthesize an extracellular chitinase, which would enable the organism to penetrate the aquatic host and survive in a chitin-rich environment. A 66 kDa chitinase from *Vibrio* species (Ohtakara *et al.*, 1979) and a 90 kDa chitobiase from *V. harveyi* (Jannatipour *et al.*, 1987) have been described. A number of the *Vibrio* strains examined by Oishi *et al.* (1979) expressed phytohemagglutinin-like agglutinins in the medium. Expression of hemagglutinins by *Vibrio cholera* is well documented (Cuatrecasas, 1973; Oishi *et al.*, 1979; Myatt and Davis, 1989). Proteases ranging from 34,500 Da to 43,000 Da have been observed in *V. cholera* (Young and Broadbent, 1982). *V. proteolyticus* secretes a 34,800 Da protease, as reported by Durham (1990). Hemolysins with masses of 40,000 to 45,000 Da have been reported from *V. hollisae* and *V. parahaemolyticus* (Reyes *et al.*, 1983: Yoh *et el.*, 1989). A different, 118 kDa hemolysin was described in *V. parahaemolyticus* by Sakurai *et al.* (1973). The expression in *V. alginolyticus* of an inducible collagenase, of undescribed molecular mass, was reported by Reid *et al.* (1980). Two copper-induced copper-binding proteins (26,000

and 28,000 Da) have been observed in *Vibrio alginolyticus* (Harwood-Sears and Gordon, 1990).

2.4 Enzyme induction

Generally, the genes coding for the particular enzymes in a pathway are grouped in specific operons in bacteria. The enzymes for any particular pathway are controlled together by the action of small molecules called inducers and repressors. The sole function of the repressor is to impede the expression of these enzymes by binding with high affinity to the operator region of the particular operon, thus preventing RNA polymerase from initiating transcription at the promoter. The repressor, coded by a regulator gene, is specific for a particular inducible operon. Apart from binding the operator, a repressor has a binding site for the inducer, which is usually a small molecule. The repressor-inducer complex is not capable of binding the operator, thus allowing RNA polymerase to transcribe the genes that form the operon (Lewin, 1987).

Generally, induction of chitinase in bacteria has been effected by chitin (Monreal and Reese, 1968; Roberts and Cabib, 1982). In *Beauveria bassiana*, chitinase was reportedly induced by glucosamine, N-acetylglucosamine and di-N-acetylchitobiose (Smith and Grula, 1983). Di-N-acetylchitobiose is a strong enzyme inducer in *Vibrio harveyi* (Soto-Gil and Zyskind, 1984).

2.5 The bacterial phosphotransferase system

The uptake of carbohydrates by bacteria may proceed through the phosphoenolpyruvate:glycose phophotransferase system (PTS) (Mitchell *et al.*, 1982) or by a simpler, proton-driven symport mechanism as for the case of the cellular import of lactose (Meadow *et al.*, 1990). The PTS catalyzes the phosphorylation of some sugar substrates (PTS sugars) concurrent with their translocation across the bacterial cell membrane. The system consists of HPr and enzyme I (EI), 2 cytoplasmic proteins that are common to the translocation of almost all PTS carbohydrates, and 2 sugar-specific proteins. After a series of transfer events, the phosphoryl group of the donor molecule phosphoenolpyruvate (PEP) is finally transferred to the sugar by a specific permease protein. The main import of glucose, which is one of the PTS sugars, is mediated by the cytoplasmic enzyme $EIII^{Glc}$ and the glucose-specific transmembrane permease EII^{Glc}. $EIII^{Glc}$ (encoded by the *crr* gene), which is responsible of phosphorylating the permease, controls the uptake of other, non-PTS sugars by inhibiting the particular permeases and by regulating the concentration of cAMP. The expression of the genes coding for the sugar-catabolyzing enzymes is usually dependent on the binding of cAMP/CAP. In the phosphorylated state, $EIII^{Glc}$ acts as an activator in the synthesis of cAMP (Saier, 1989). When not phosphorylated, this PTS protein binds to and inhibits the activity of adenylate cyclase. The PTS is also involved in the chemotactic response of bacteria (Roseman and Meadow, 1990).

The bacterial phosphotransferase system has been best characterized for the enteric bacteria *Escherichia coli* and *Salmonella typhimurium*, but is widely distributed among other terrestrial, pathogenic and freshwater bacteria. Among the marine bacteria, a homologous phosphotransferase system has been described for several species of the *Vibrio* genus (Meadow *et al.*, 1987). Studies among Gram-positive bacteria have resulted in the conclusion that their PTS is very similar to the PTS in Gram-negative bacteria (Meadow *et al.*, 1990).

2.6 Bacterial protein secretion

The final destination of proteins upon translation is encoded in their primary sequence. In Gram-negative bacteria, secreted proteins must cross the inner membrane, the peptidoglycan-containing periplasm, and the outer membrane. It has been proposed that multiple mechanisms of protein export may exist for different proteins, even in the same cell (Dow *et al.*, 1989). In *Escherichia coli*, most periplasmic and outer membrane proteins are synthesized as precursors containing an NH_2-terminal sequence which is cleaved by a leader peptidase (LP) and which is not found in the mature protein (Pugsley and Schwartz, 1985). This region, called the signal peptide, spans generally from 15 to 40 residues and exhibits some essential characteristics: one or more positively charged residues at the extreme amino-terminus, an unbroken stretch of 8 or more hydrophobic or neutral residues

with a strong tendency to form an α-helix, and a signal peptidase cleavage site. Proteins containing a glyceride-fatty-acid modified Cys residue immediately after the cleavage site are processed by a lipoprotein signal peptidase (LSP). Proteins transverse the membrane through pores, formed by integral membrane proteins (Pugsley and Schwartz, 1985). Other models consider binding of protein precursors to the hydrophobic environment of the membrane (Tommassen *et al.*, 1989). Some secreted proteins, like haemolysin in *E. coli* and ß-lactamase in *Salmonella typhhimurium* do not contain an NH_2-terminus signal peptide. Their export is mediated, principally, by information contained in the C-terminal region (Koshland and Botstein, 1980).

2.7 Aim of the study

In the isolates of *Vibrio parahaemolyticus* used in this study, chitin induces several chitin-binding proteins that are released onto the culture supernatant fraction: a 95 kDa chitinase (that may degrade to a 65 kDa chitinase), a chitobiase with an apparent mass of 80 kDa, and a third protein, of unknown function, with an approximate molecular mass of 134 kDa.

The study on the isolation, purification and characterization of a chitinase enzyme from *Vibrio parahaemolyticus* was initiated having a practical goal in mind: to obtain large amounts of a chitin-hydrolase to be used in chitin-waste processing plants, in order to generate products useful in the pharmaceutical industry and as

fish- and animal-feed. Thus, it was important to determine whether the enzyme exhibited sturdy and robust characteristics needed in an industrial biodegradation setting.

During the purification of the chitinase from *Vibrio parahaemolyticus*, a contaminant protein was discovered to have chitin-binding properties. The purification and partial characterization of this novel chitin-binding protein was attempted for two reasons: i) chitovibrin appears to be an uncharacterized bacterial protein, and ii) the chitin-binding capacity of the protein suggests it may be one of the few bacterial lectins known so far.

The present report focuses on the isolation and characterization of the 95 kDa form of the chitinase and on the 134 kDa protein that was tentatively named *chitovibrin* .

Chapter 3

Materials and Methods

3.1 Organisms and materials

3.1.1 Organisms

The wild-type *Vibrio parahaemolyticus* isolate (ATCC 27969), as well as a mutant strain (L101)* were normally grown on 804 medium (0.75 g/l KCl, 6.9 g/l $MgSO_4$, 23.4 g/l NaCl, 1 g/l tryptone, and 1 g/l yeast extract) supplemented with 0.5% pretreated commercial chitin. The time of harvest (when the chitin dissappears from the medium, and generally assessed visually) was between 2.5 and 4 days after inoculation for the wild-type strain. The disappearance of the chitin in cultures containing the mutant organism occurred only 15 hours after inoculation. The L101 mutant expressed the same proteins as the wild-type upon induction with chitin but the production of proteins apparently was not affected by the presence of other nutrients in the growth medium. The isolates were maintained in small plastic vials at -70°C in 804 medium (see Appendix A1) with 30% glycerol.

3.1.2 Materials

The Protean II minigel system, the protein assay mixture, as well as the ion-exchange and gel filtration resins were purchased

* kindly provided by Dr. Ronald Siebeling, Department of Microbiology, Louisiana State University, LA

from Bio-Rad (Richmond, CA). Chitin, chitosan, buffers, electrophoresis standards, dipalmitoylphospatidylethanolamine (PPEADP), and p-nitrophenyl butyrate were purchased from Sigma Chemical Company (St. Louis, MO). Acrylamide:bisacrylamide solution (37.5:1) was from Amresco (Solon, OH). The model 59A microcentrifuge, a waterbath with shaker, and disposable C-18 columns were from Fisher Scientific (Fair Lawn, NJ). The ^{3}H-acetic anhydride and the Na^{125}I were purchased from ICN Biomedicals (Costa Mesa, CA). The Iodo-beads used in radioiodination of the proteins were purchased from Pierce (Rockford, IL). The GS 300 scanning densitometer and the gel dryer were from Hoefer Scientific Instruments (San Francisco, CA). Fraction collectors were from Gilson (Middleton, WI). The microdialysis systems were from BRL (Gaithersburgh, MD). The silica gel G plates used for the thin-layer experiments were purchased from Analtech (Newark, DE). Sepharose 4B, 6B and Sephadex G-75 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The cell disruptor was from Heat Systems-Ultrasonics, Inc. (Farmingdale, NY). Positive/Negative instant negative film 55 and XAR5 Diagnostic Film were purchased from Polaroid, Inc. (Cambridge, MA) and Eastman Kodak Co. (Rochester, NY), respectively.

3.2 Methods

3.2.1 Pretreatment of chitin (swollen chitin)

When supplementing the growth media with chitin, the substrate from commercial sources was treated with acid to swell it

and make it more easily digestable by the bacteria. Swollen chitin was prepared from crab chitin (Sigma, practical grade) and phosphoric acid, following the procedure by Monreal and Reese (1968). After mixing in a blender with concentrated phosphoric acid at a concentration of 1 g chitin /10 ml acid, the chitin was incubated for 2 days at 4°C and slow stirring. After the incubation, the viscous material was carefully neutralized with 2N NaOH. The swollen chitin was recovered by slow speed centrifugation and washed extensively, first with running, then with distilled water. The washed chitin was kept refrigerated in 2 mM sodium azide. Before adding this chitin to any media, the preservative was removed by washing and centrifuging.

3.2.2 Measurement of chitinase activity

A simple method of measuring chitinase activity is to incubate the enzyme with [^{3}H]-chitin and measuring the radioactivity of soluble products in the supernatant fraction after the incubation. The method is essentially the one described by Molano *et al.* (1977) and Cabib (1988), with minor modifications. A volume of 190 µl of tritiated substrate (~90,000 cpm/µmole GlcNAc) is incubated in a microvial with 10 µl of the enzyme preparation in a water bath with shaker at 45°C. After 10 minutes the reaction is stopped by the addition of 200 µl of 20% trichloroacetic acid. The mixture is centrifuged in a microcentrifuge at maximum speed for 2 minutes, and 100 µl of the clear supernatant are assayed for tritium activity. It is convenient to exchange the liquid phase from the chitin

substrate suspension before incubation if the background activity is to be kept low. This is accomplished by removal of the supernatant liquid after a 2 minute centrifugation of the suspension, and the addition of an equal volume of a buffer with the desired pH.

3.2.3 Endochitinase activity

To test for endo- or exo-glycosyl hydrolase activity, 1.3 µg purified chitinase was incubated with 4-methylumbelliferyl-N,N',N''-triacetyl-ß-chitotrioside (5 µM) and the reaction rate monitored by fluorescence at 440 nm, according to Robbins *et al.* (1988). The total volume was 2 ml and the reaction mixture contained 25 mM buffer (Pipes, pH 6 or Bicine, pH 8). The experimental procedure was performed by Milligan C. Fossett, in our laboratory at that time.

3.2.4 Tritiated chitin

The tritiated chitin used as substrate for chitinase activity measurements was prepared by reacetylation of chitosan with [^{3}H]-acetic anhydride, according to reported procedures (Cabib, 1988). Before reacetylation, the commercial chitosan (Sigma) usually had to be further deacetylated in order to make it more reactive. The deacetylation procedure used was by Mima *et al.* (1983). 2 g of Sigma chitosan were incubated in 40 ml 5% acetic acid for 4 hours in the cold. The undissolved particles were separated by fitration through cheesecloth and the viscous liquid squirted slowly into a larger volume (1 liter) of stirring 1 N NaOH. The chitosan, which precipitates at high pH, was separated in a Buchner funnel and

transferred to a 400 ml beaker filled with 47% NaOH in water at 90-95°C. The chitosan was incubated in the NaOH solution under N_2 for 4 hours, and then filtered and washed extensively. A fine chitosan powder was obtained by lyophilization. After redissolving 1 g of this chitosan in 20 ml of 10% acetic acid, methanol was added to a final volume of 200 ml. Fifty microliter tritiated acetic anhydride (1 mCi) was added slowly to the stirring solution of chitosan under a vented hood. After 10 minutes incubation, unlabeled acetic anhydride was added until the material gelified. The gel was cut into small pieces to let the trapped solution drain. After sitting for 1 hour, the liquid was decanted, and the solid washed and homogenized in a blender. The specific activity of the preparation was obtained by measuring the activity of known volumes and weights of the chitin slurry, and it was normally around 0.5 µCi/mg chitin, corresponding roughly to 0.1 µCi/µmole GlcNAc.

3.2.5 Thin-layer chromatography

Thin-layer chromatography was performed on prescored silica gel G plates, which were developed with a proportion of acetonitrile:H_2O varying from 2:1 to 4:1, depending on the size of the oligosaccharides being separated. The visualization of the chitin oligomers was obtained by charring of the plates at 140°C after spraying with 50% sulfuric acid. Standards from the N-acetylglucosamine monomer to the hexamer were commercially available from Seikagaku (Ft. Lauderdale, FL). Higher oligomers (up

to 12-mer) were prepared in the laboratory from acid hydrolysates of chitin as described later.

3.2.6 Antichitinase antibodies

For an effective purification of chitovibrin, the removal of the chitinase -especially the 95K form of this enzyme- was accomplished by passing the protein concentrate on a Sepharose-chitinase antibody affinity gel column. Rabbits were inoculated with cloned chitinase and the antiserum was purified as described by Chua *et al.* (1982) and by Maurer and Callahan (1980). The anti-chitinase IgG were affinity purified on a Sepharose-chitinase gel column and later covalently attached to a Sepharose 4B matrix after activation with cyanogen bromide according to the procedure described by Dean *et al.* (1985). The cloned chitinase preparation is devoid of chitovibrin.

3.2.6.1 Production of chitinase antiserum

Three white New Zealand rabbits were inoculated subcutaneously, each with 1 mg of cloned chitinase emulsified in 1 ml H_2O:complete Freund's adjuvant (1:1). The rabbits were boosted after 2 weeks with 0.5 mg of the same protein. For the booster injections, the emulsion was prepared with incomplete Freund's adjuvant. The bleeding was performed 2 weeks after the boost and every week thereafter. Every 3 months the rabbits received a booster shot of chitinase in incomplete Freund's. Each rabbit produced between 25 and 40 ml of blood every week. The blood was incubated at 37°C for 1 hour. The formed clot was cut into small

pieces. After the incubation, the blood was centrifuged for 20 minutes at 2,000 x *g* and the clear serum was removed, aliquoted and stored at -20°C.

3.2.6.2 Purification of antichitinase antibodies

40 ml of rabbit antichitinase antiserum were diluted with an equal volume of water and precipitated by the addition of ammonium sulfate to 36% saturation. The pellet obtained by centrifugation at 10,000 x *g* for 10 minutes was redissolved in 36% ammonium sulfate and repelleted under the same previous conditions. The pellet was dissolved in 10 mM Tris-HCl buffer pH 7.5 and dialyzed extensively against the same buffer. The solution was then loaded onto a column of Sepharose-chitinase gel and washed with 10 mM Tris buffer. Bound antibodies were eluted with 6 M guanidine-hydrochloride. The antibodies bound to the Sepharose-chitinase gel recognized chitinase specifically and could be used for the purification of chitovibrin or in Western blot analysis of the *Vibrio* proteins.

3.2.6.3 Immobilization of antichitinase antibodies

A 22 ml preparation of affinity-purified antichitinase antibodies (0.439 mg/ml) were immobilized onto 50 ml of Sepharose 4B gel matrix activated with 1.4 mg of cyanogen bromide as described by Dean *et al.* (1985). After the binding, the gel was incubated with 100 ml of 1 M glycine to block the remaining active sites. Prior to use, the gel was extensively washed with 10 mM

phosphate buffer, pH 6. Through analysis of protein content of original preparation and solutions after the binding step, it was determined that practically all the antichitinase antibodies were immobilized onto the Sepharose matrix.

3.2.6.4 Immobilization of chitinase

5 mg of cloned chitinase were dissolved in 20 ml of 0.1 M sodium bicarbonate and attached to 25 ml of Sepharose 4B by the cyanogen bromide activation procedure described above. The washed Sepharose-chitinase gel was packed on a column (1.5 x 3.5 cm) and used to affinity-purify antichitinase antisera.

3.2.7 Preparation of chitin oligomers

Chito-tetramer, -pentamer and -hexamer were purchased from Seikagaku (Ft. Lauderdale, FL). Larger chitin oligomers, not commercially available, were prepared by hydrolysis of chitin, either produced with hydrogen fluoride (Defaye and Gadelle, 1982 and Bosso *et al.*, 1986) or hydrogen chloride (Rupley, 1964). Before hydrolysis with HF, commercial chitin was incubated with concentrated phosphoric acid for 2 days at 4°C and later neutralized with sodium hydroxide. The slurry was centrifuged, washed thoroughly and dried. The reaction of chitin with HF was carried out at temperatures below 20°C and in sealed Teflon reaction vials. The size of the oligomers after hydrolysis depended on the time of the reaction and the temperature used. Four ml of HF were used for every gram of chitin. After letting the reaction proceed for 1 hour,

the viscous solution is precipitated in ether at -70°C. The precipitate was washed with clean ether several times and then air-dried. The solid was suspended in H_2O and filtered. The filtrate was concentrated and dried in a lyophilizer to a solid product. When hydrolized with HCl, non-treated chitin was used because the main impurities (amino acids and salts) were removed during the first 2 steps of elution from activated charcoal, used to trap the oligomers after dilution of the hydrolysate. Chitin oligomer preparations were first fractionated on a Bio-Gel P-6DG desalting gel column (3.5 x 83 cm). The fractions were eluted with 50 mM ammonium acetate to minimize the interaction between the oligomers, which tend to form clusters under low ionic strength conditions (Bosso *et al.*, 1986). A finer purification of the different oligomers was accomplished by chromatography on a Bio-Gel P-4 gel column (1 x 113 cm). This additional step was repeated in the case of the purification of larger oligomers.

3.2.7.1 Immobilization of chitin oligomers

After fractionation of the chitin hydrolysate on the Bio-Gel P-6DG column, fractions containing higher oligomers were pooled and bound to aminobutylamino-agarose by reductive amination. Twenty mg of high molecular weight chitooligomers (MW ranging from 1,000 to 3,000) were resuspended in 0.2 M borate buffer, pH 9.5 and incubated for 42 hours at 50°C with 6 ml of aminobutylamino-agarose gel and 25 mg sodium cyanoborohydride. After the incubation, the remaining cyanoborohydride was removed by

acidification with 0.5 M HCl under a vented hood. The boric acid was removed by washing with methanol. Before use, the gel was washed extensively with 10 mM phosphate buffer, pH 6.

3.2.8 Localization of chitovibrin in Vibrio parahaemolyticus

To localize chitovibrin in the growing *Vibrio* cell, the method described by Neu and Heppel (1965) and Nossal and Heppel (1966) was used. Proteins were isolated from the supernatant fraction, the hypertonic fraction, the osmotic shock fraction, and the cytosol. *Vibrio paarahemolyticus* was grown in 500 ml Erlenmeyer flasks with 804 Medium + chitin (4 g/l) and harvested after 15, 20, 25 and 30 hours of incubation at 37°C. The removal of the supernatant fraction was by centrifugation, and the cells were suspended in 100 ml of 20% sucrose-30 mM Tris-HCl, pH 8. Upon the addition of EDTA to a concentration of 1 mM, the cells were incubated at 24°C with stirring. After 10 minutes, the cell suspension was centrifuged for 10 minutes at 13,000 x *g* in the cold. The supernatant fluid was termed the hypertonic fraction. The cell pellet was rapidly mixed with 100 ml of cold water and incubated with stirring over ice. The cells were separated by centrifugation and the supernatant solution was kept as the hypotonic fraction. The cells were subjected to sonic disruption (5 x 1-second pulses) after resuspension in 100 ml Tris-HCl buffer (10 mM pH 8), made 1 mM in EDTA. The suspension was centrifuged under same conditions, and the supernatant fluid stored as the cytosolic fraction. The proteins from the different solutions were

recovered by centrifugation for 30 minutes at 20,000 x *g* after the addition of ammonium sulfate to 70 % saturation and incubation for 8 hours.

3.2.9 Preparation of neoglycolipids

The procedure was that described by Stoll *et al.* (1988), with some modifications due to the low solubility of the chitin oligomers in non-aqueous solvents. Essentially, 5 µmoles of lyophilized oligomer were dissolved at 65°C in 0.5 ml of a 5% solution of lithium chloride in N,N'-dimethylacetamide (DMAc) (McCormick and Lichatovich, 1979). Dissolution in aqueous solvents of lyophilized chitin oligosaccharides with degree of polymerization higher than 7 is a slow process, as was observed during this study. Total solubilization of the oligosaccharides was obtained upon incubation in 5% lithium chloride-N,N'-dimethylacetamide for 12 hours at 50°C. The oligomer solution was mixed with 5.24 ml of a 5 mg/ml solution of dipalmitoylphospatidylethanolamine in chloroform:methanol (1:1). The mixture was sonicated for 10 minutes in a sonic bath and incubated for 2 hours at 65°C in a reaction vial sealed wih a Teflon-lined cap. Then, 1.2 mg of sodium cyanoborohydride in methanol (120 µl) were added, and the mixture was further incubated at 65°C for 16 hours. The excess of cyanoborohydride was destroyed by the addition of a few drops of 0.5 M HCl in a ventilated hood. After the removal of the cyanoborohydride, the sample was neutralized with ammonium bicarbonate and the boric acid was co-evaporated with methanol under nitrogen atmosphere. The dried product was

redissolved in chloroform:methanol:H_2O (15:70:30) (Solvent A-L) and applied to a disposable silica-C18 column. The column was washed with the same solvent and then eluted with chloroform:methanol:H_2O (60:35:8) (Solvent B-L), according to Lawson *et al.* (1990). The binding activity of the eluted fractions was assayed with labeled wheat germ agglutinin (WGA) on a 96-well plate binding experiment.

3.2.9.1 Concentration of neoglycolipids

After chromatography of the neoglycolipids on the C-18 columns, the samples still contain variable amounts of the unreacted lipid. The determination of the actual concentration of the neoglycolipids was performed through the reaction with p-dimethylaminobenzaldehyde (DMAB), as described by Reissig *et al.* (1955), with minor modifications. This procedure is used to determine hexosamines. For the assay, 100 µl aliquots of the samples were allowed to dry at room temperature and then incubated with 95 µl of H_2O and 5 µl of a chitobiase preparation at 37°C for 4 hours. After the addition of 20 µl of 0.8 M potassium tetraborate, pH 9.5, the samples were boiled for 3 minutes. After cooling down to room temperature and adding 750 µl of freshly prepared DMAB reagent, the samples were incubated at 37°C for 30 minutes, after which the absorbance at 585 nm was measured. A calibration curve was prepared using samples with known concentrations of chito-4mer.

3.2.10 96-well plate binding assay

Samples of the neoglycolipid solutions were poured into different wells and dried at room temperature (RT). The wells were then incubated at RT for 2 hours on the shaker with 300 µL of 1.5% BSA. After this incubation, the wells were rinsed with dH_20 and incubated on the shaker at RT for 2 hours with the labeled (usually ^{125}I) protein to be tested in a 1.5% BSA solution. A total of 60,00 to 100,000 cpm of ^{125}I in 300 µl were applied to each well. The labeled protein was carefully removed and disposed of, and the wells were incubated for 20 minutes with 250 µL PBS and then rinsed with dH_20. After the rinse, 200 µl of 6M guanidine-HCl were added to each well and incubated at RT for 10 minutes. The guanidine-HCl was removed and tested for label.

3.2.11 Binding of chitovibrin to chitin

One hundred µl of a suspension in H_2O of regenerated chitin, which was about 2 g/l, was centrifuged for 2 minutes in a minifuge. After removing the supernatant fraction completely and adding 50 µl of the buffer at the desired pH, the suspension was mixed by vortexing for a few seconds. An aliquot of the radiolabeled chitovibrin was added and the sample was vortexed again slightly, and then incubated in the cold for 30 minutes. After spinning again and carefully discarding the supernatant liquid, the remaining solid was rinsed with H_2O. After a new centrifugation, the pellet was boiled with loading buffer and 2-mercaptoethanol, and then subjected to electrophoresis. The gel was stained, destained and

dried. After obtaining the autoradiography, the relative binding could be assessed through densitometry performed on the film.

3.2.12 Electroblotting of proteins

The transfer of proteins was performed onto polyvinyledene fluoride membranes as described by Matsudaira (1987 and 1990). Proteins were electrophoresed on a 6% SDS polyacrylamide gel after a 20 minute prerun with sodium thioglycolate, to prevent blocking of the proteins. The transfer was accomplished on an transfer apparatus where gel and membrane are sandwiched between layers of filter paper, at 40V and a total of 0.68 Ampere-hour.

3.2.12.1 Western-blot analysis

The proteins were tested for binding to specific antibodies after transfer to the membrane. The bound antibodies were visualized by autoradiography upon incubation with radioiodinated *Staphylococcus* protein A. The radiographic detection procedure was taken directly from Burnette (1981). The protein-containing membranes were incubated with diluted (1:100) antiserum for 90 minutes, treated with labeled protein A, and allowed to expose X-ray film at -70°C for 3-18 hours.

3.2.13 Column chromatography

3.2.13.1 Chitin column chromatography

To test for the binding to chitin, the protein preparations obtained from the *Vibrio* isolate were passed through a 1 ml column

prepared with regenerated chitin, in which the commercial chitosan from Sigma was further deacetylated (Mima *et al.*, 1983) and then reacetylated following the procedure by Cabib (1988) described above.

3.2.13.2 Size-exclusion chromatography

For separating proteins by size, columns packed with Bio-Gel P-150 and P-200 gel (1.5 x 120 cm) were used. The columns were calibrated with thyroglobulin (669 K), BSA (67 K), ovalbumin (43 K) and carbonic anhydrase (29 K), and generally eluted with 10 mM potassium phosphate buffer, pH 6 (KP buffer). Chitin oligosaccharides were first coarse-purified on a P-6DG desalting-gel column (4.5 x 42 cm). Further purification was accomplished on a Bio-Gel P-4 gel column (0.8 x 85 cm).

3.2.13.3 Hydrophobic interaction chromatography

A 3 ml bed of Phenyl-Sepharose-CL 4B was packed into a column (0.8 x 6 cm). Samples were prepared by increasing the salt (usually sodium chloride) concentration to 4 M and loaded onto the column, equilibrated with a 4 M-salt buffer. Samples were eluted with gradients with decreasing salt concentration at room temperature or in the cold (Kennedy, 1990). Samples de-associate easier from hydrophobic gels at lower temperatures.

3.2.14 Radiolabeling of proteins

The proteins were iodinated by the widely used method involving the oxidation of $Na^{125}I$ in the presence of tyrosine residues. In the case of chitovibrin, the protein sample consisted of 100 µl of a 0.18 mg/ml preparation incubated for 5 minutes with 1 mCi of $Na^{125}I$ in the presence of 2 Iodo-beads in 100 µl buffer, pH 7.5 (10 mM Tris-HCl). The preparation was chromatographed on a Pharmacia PD-10 prepacked column with a 9.1 ml bed of Sephadex G-25 M gel. The bed height was 5 cm, and the volume of the fractions collected was 0.6 ml. To monitor the label on each fraction, 3 µl samples were analyzed for ^{125}I. The final specific activity was about 3.2×10^6 cpm/µg protein.

3.2.15 TCA-Filter assay

Samples of labeled proteins were aliquoted on nitro-cellulose filters (Whatman, 3 mm) and left to air-dry. Controls were measured for radioactivity. TCA (20%) was pipetted over the rest of samples which were left to dry in air. Filters were washed in ether-ethanol (1:1) for 10 minutes and in ether for 30 minutes, after which the filters were left to dry and later measured for radioactivity.

3.2.16 IEF experiments

The gels for the IEF experiments were prepared with 2.8 g sucrose, 3.12 ml 40% acrylamide/bis (37.5:1), 2 ml of ampholines (pH 3-7), 80 µl ammonium persulfate and H_2O to 25 ml. The polymerization was initiated by the addition of 10 µl of TEMED. The

pH standard consisted of a mixture of 7 pH markers, from pH 3.6 to 6.6 . The gel was placed on a flat-bed, cooled to 0°C and prerun for 30 minutes at 4 W. After adding the sample, the electrophoresis normally took between 5 and 6 hours at 4 W. The pH of the electrodes was maintained by placing them on thick filter paper soaked in 0.1 M NaOH or H_2PO_3. After electrophoresis, the gel was washed extensively (for 24 hours, with 2 changes of PBS) to remove the ampholines. The staining and destaining was done as for regular Laemmli (1970) gels.

3.2.17 Induction of protein by different saccharides

To test for the capability of other carbohydrates to produce a similar inducing effect, the organism was grown in media containing different saccharides. Thirty five ml cultures were grown in 125 ml Erlenmeyer flasks with 804 M or M9CA (minimal) medium supplemented with the sugars at a concentration of 0.57 mg/ml. After 10 hours, the cultures were harvested, and the supernatants analyzed for chitinase and chitobiase activity after precipitation with 50% ammonium sulfate, and dialysis of the proteins. The different protein bands were analyzed by electrophoresis on 6 % SDS-polyacrylamide gels.

3.2.18 General methods

Protein content was determined by the method described by Bradford (1976), using 1 ml of a 5x diluted Bio-Rad protein assay mixture and 100 µl of sample. The SDS-PAGE experiments (usually

7.5% or 6%) were done according to Laemmli (1970). The gels were stained for 30-60 minutes (depending of the size of the gel) with 0.1% Coomassie Blue R in 50% methanol in H_2O. The destaining was produced with several changes of a methanol/acetic acid/H_2O (2:1:7) solution.

Chapter 4

Characterization of chitinase from *Vibrio parahaemolyticus*

4.1 Introduction

The first experiments were done with cultures of a wild-type *Vibrio parahaemolyticus* (ATCC 27969) kindly provided by Dr. Ronald Siebeling*. The organism normally was grown on 804 medium (0.75 g/l KCl, 6.9 g/l $MgSO_4$, 23.4 g/l NaCl, 1 g/l tryptone, and 1 g/l yeast extract) supplemented with 0.5% pretreated commercial chitin.

The time of harvest, (when the chitin dissappears from the medium, and generally assessed by visual observation) was between 2.5 and 4 days after inoculation. After continously passing the organism on agar plates the proteins in the culture filtrates as well as the chitinolytic activity decreased sharply. We obtained several new strains of *Vibrio parahaemolyticus* isolated from oysters in Dr. Siebeling's laboratory. One of the isolates (L101), apparently a mutant strain, could be harvested only 15 hours after inoculation.

The L101 isolate expressed the same proteins as the wild-type upon induction with chitinase but the production of the

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proteins apparently was not affected by the presence of other nutrients in the growth medium. The isolates were maintained in small capped plastic vials at -70°C in 804 M (see Appendix A1) with 30% glycerol.

4.2 Isolation and purification of chitinase from *Vibrio parahaemolyticus*

Chitinase from *Vibrio parahaemolyticus* was successfully cloned into *Escherichia coli* by Dr. Chin-Yih Ou, then in our laboratory. Enzyme characterization studies were possible, in part, due to the availability of a cloned enzyme.

Chitinase is a chitin-inducible enzyme in *Vibrio parahaemolyticus*. When growing the bacteria on 804 medium alone, there was only a low level of expression of the enzyme to the growth medium. When medium was supplemented with chitin, protein bands were apparent in the growth medium 36 hours after inoculation, as analyzed on SDS-polyacrylamide gel electrophoresis (*Figure* 4.1-A). Concomitantly, a peak of chitinase activity was detected as shown in *Figure* 4.1-B. The disappearence of chitinase activity and protein bands after 48 hours incubation may be explained by the adsorbance of the enzyme to chitin in the medium. The binding of the 2 forms of chitinase was apparent upon incubation of a crude enzyme sample with regenerated chitin, as shown in *Figure* 4.2.

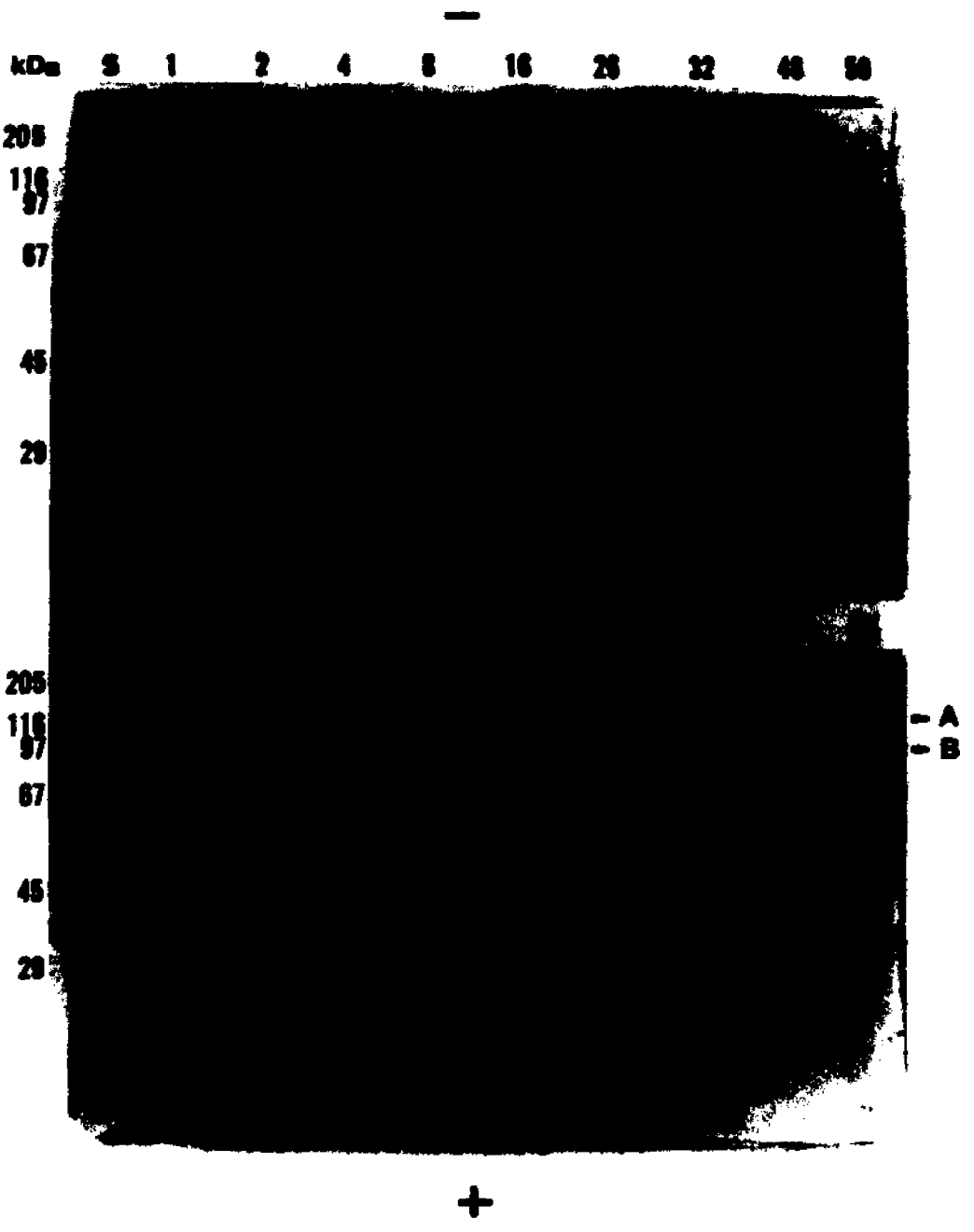


Figure 4.1-A **Growth of *Vibrio parahaemolyticus* (I). Time-course analysis of the proteins secreted to medium, supplemented (+) or not (-) with chitin. Proteins were analyzed on 7.5% SDS-PAGE. The sample on each lane corresponds to 100 µl supernatant fraction. Numbers on top of figures correspond to hours of incubation. S = high MW standards. A= chitovibrin; B= chitinase.**

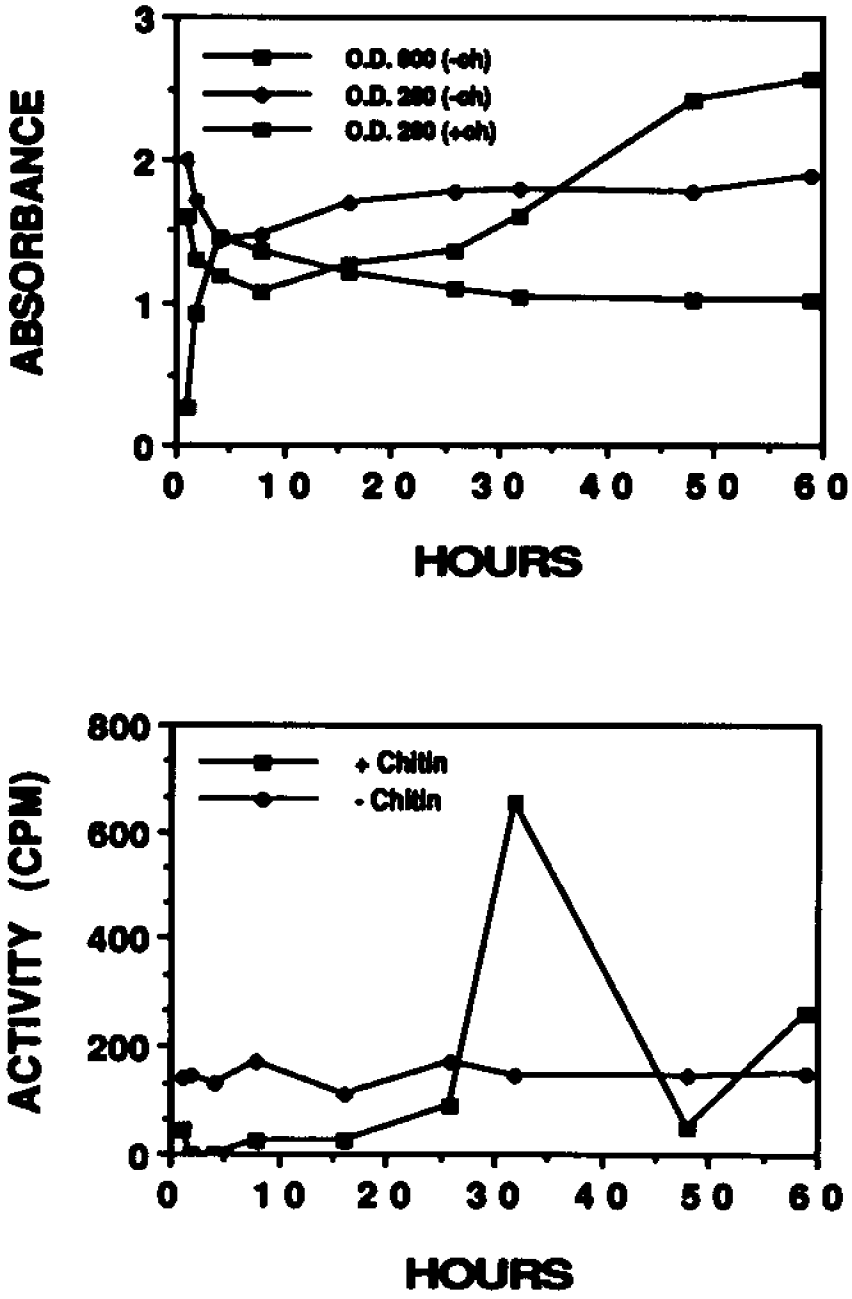


Figure 4.1-B Growth of *Vibrio parahaemolyticus* (II) Absorbance and chitinase activity of fractions at different sampling times for cultures with chitin (+) or no chitin (-) added. Activity, expressed in cpm was determined for 100µl samples (see Methods).

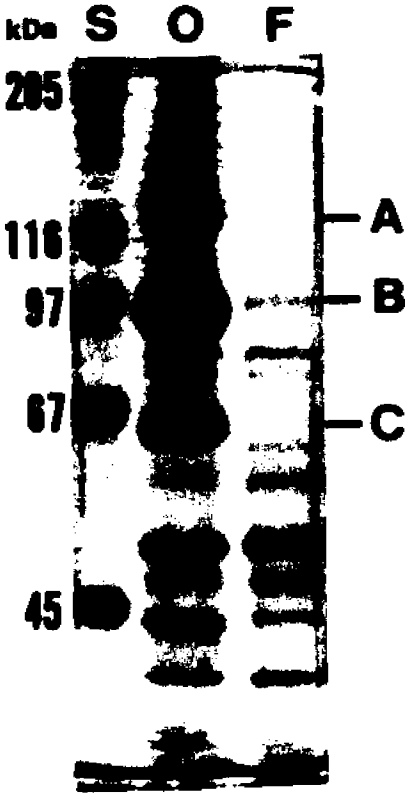


Figure 4.2 **Binding to chitin column.** A 2 ml (3 mg) sample of crude extract was passed through a 2 ml (wet volume) chitin column. Proteins from original (O) and flowthrough (F) fractions were analyzed on 6% SDS-PAGE. A= chitovibrin; B = 95K chitinase; C = 65K chitinase; S = High MW standards.

4.2.1 Ammonium sulfate precipitation, chitin-affinity and anion-exchange chromatography

When chitin had disappeared from the growth media, cultures were centrifuged (5,000 x *g*) and the cell pellet was removed. The clarified fraction was incubated at 4°C for 12 hours after the addition of 5 g of chitin/liter of solution. The chitinase was recovered after digestion of the chitin. The digestion was carried out for 2 days at 37°C and stirring upon addition of azide (1mM) and phenylmethylsulfonylfluoride (0.1 mM). The sample was centrifuged and the supernatant fraction was concentrated on a pressure filter, after which the absorbance at 280 nm was 1.7 O.D. The sample was passed through DEAE Bio-Gel A chromatography column, and eluted using a gradient from 0 to 0.3 M NaCl in potassium phosphate buffer, pH 6. The eluant was collected in 2.9 ml fractions with measurement of absorbance at 280 nm. Fractions were assayed for chitinase activity.

The results, presented in *Figure* 4.3-A, show the main chitinase peak eluting at 0.25 M NaCl. Figure 4.3-B shows a 5% polyacrylamide (SDS) gel electrophoresis assay for fractions 36, 46, 53, 62, 64, 66, 68 and 70, stained with Coomassie Blue. The samples were dialyzed, lyophilized and redissolved in 100 µl H_2O, which corresponded to a 5-fold concentration of the original fraction. Thirty microliter were loaded onto each well in the polyacrylamide gel (*Fig.* 4.3-B).

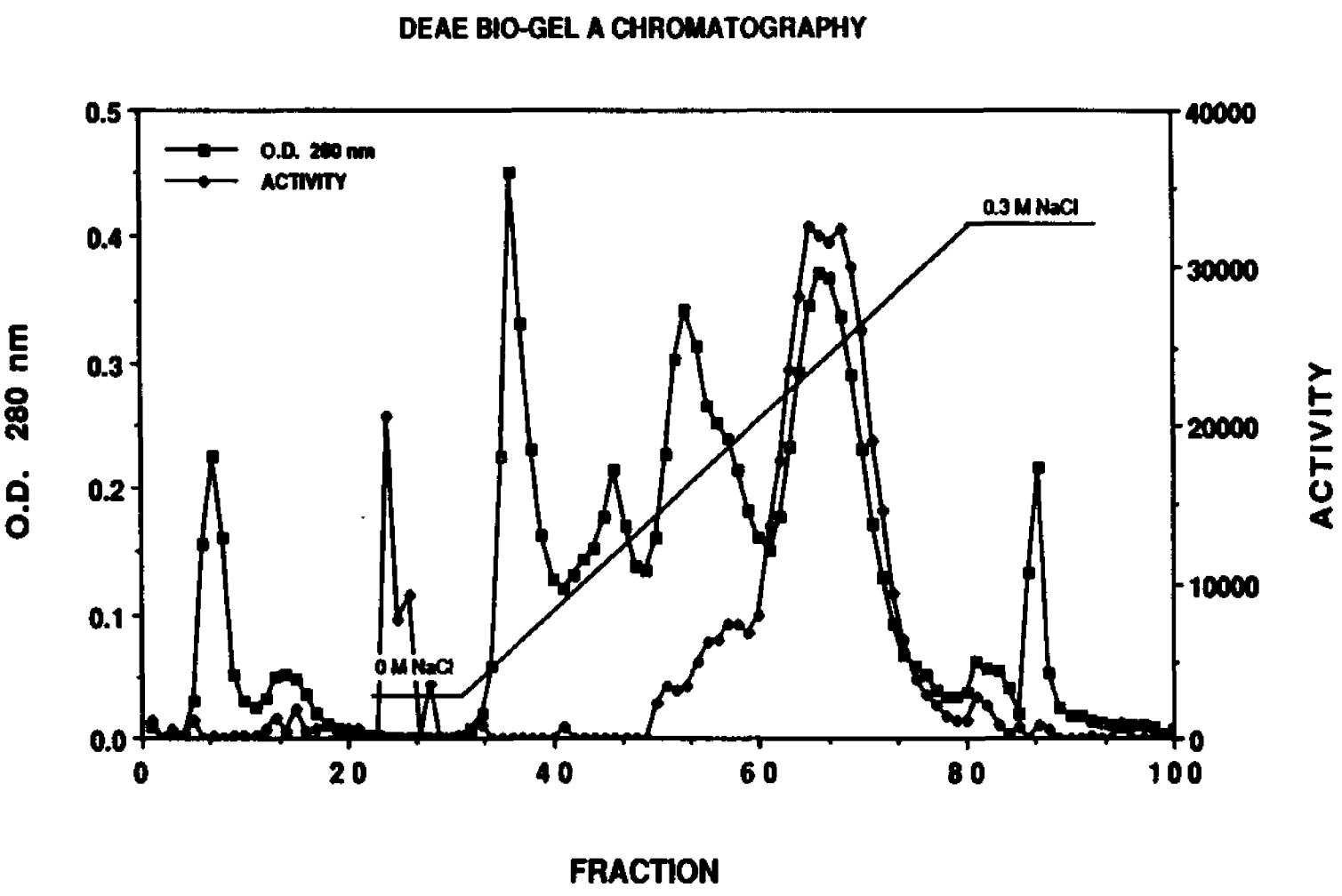


Figure 4.3-A **Ion-exchange chromatography (I).** The chitinase concentrate preparation was chromatographed on a DEAE Bio-Gel A column as described in Methods. The activity is expressed in cpm released (^{3}H); 100 µl were assayed for each fraction.

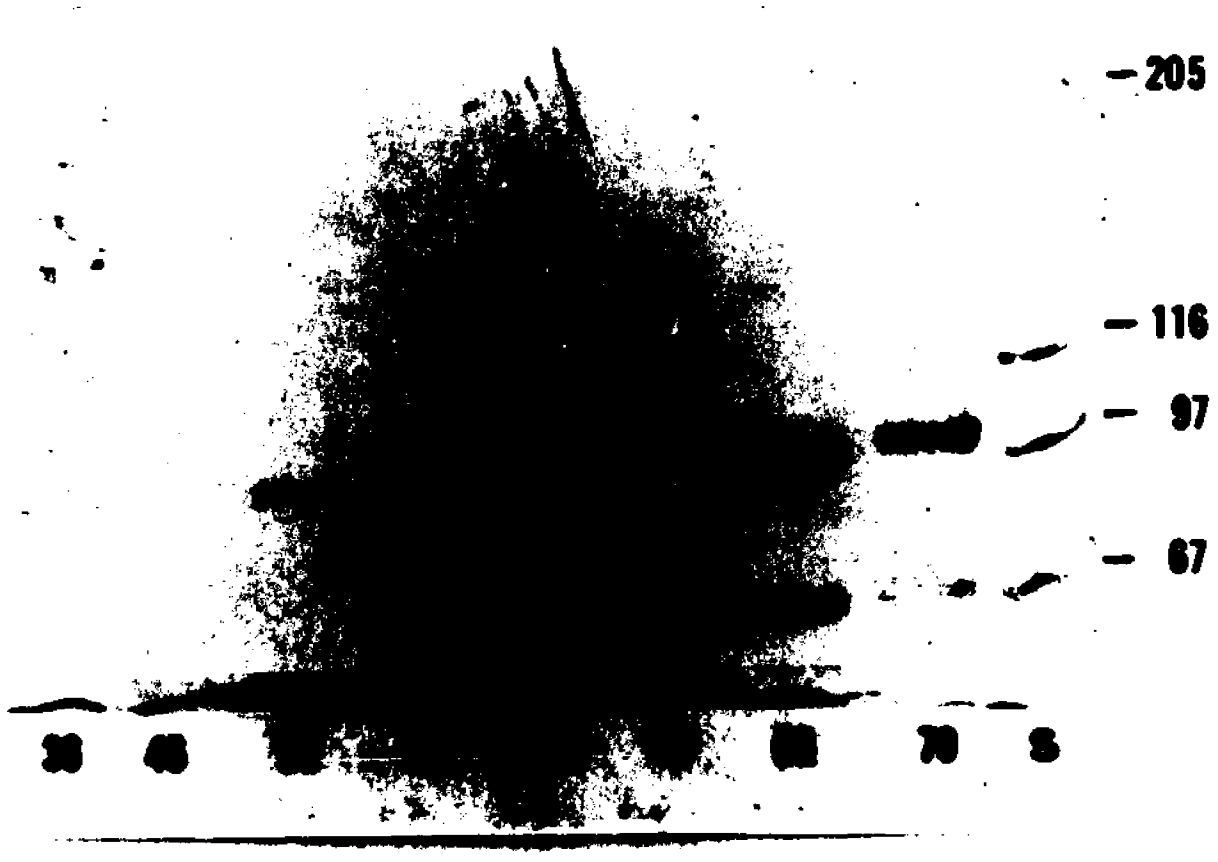


Figure 4.3-B **Ion-exchange chromatography (II). SDS-polyacrylamide gel electrophoresis (5%) assay for fractions 36, 46, 53, 62, 64, 66, 68 and 70 from DEAE Bio-Gel A elution (*Fig* 4.3-A), stained with Coomassie Blue.**

4.2.2 Gel permeation chromatography

From SDS-PAGE results on the activity peak from the DEAE column (*Fig.* 4.3-B), chitinases have apparent molecular masses of 95 kDa and 65 kDa. To separate these proteins, fractions 61-73 were pooled and lyophilized, recovering 44.2 mg of dry material which was resuspended in 2 ml H_2O. The redissolved sample was dialyzed again, and 1.9 ml loaded onto a Bio-Gel P-150 column (1.5 x 95 cm) upon addition of sucrose and dye (BPB).

Fractions of 1.45 ml of eluant were collected and chitinase activity was determined as shown in *Figure* 4.4-A. The two distinct activity peaks (fractions 44 to 49 and 55 to 61) were pooled into pool I and pool II, respectively. After lyophilization, the pools were resuspended in H_2O and electrophoresed on 7.5% SDS polyacrylamide gel as shown in *Figure* 4.4.-B. To examine the purity of the polypeptides in each pool, samples loaded onto the polyacrylamide gel contained increasing amounts of protein. From the gel electrophoresis results, the purity of the *Vibrio* chitinases was estimated to be 90 % for the 95K form, and >95 % for the 65K polypeptide.

The chitinase activity was stable to at least 5 freeze-thaw cycles and lyophilization. The activity remained constant upon addition of glycerol (10 %) to the samples (not shown).

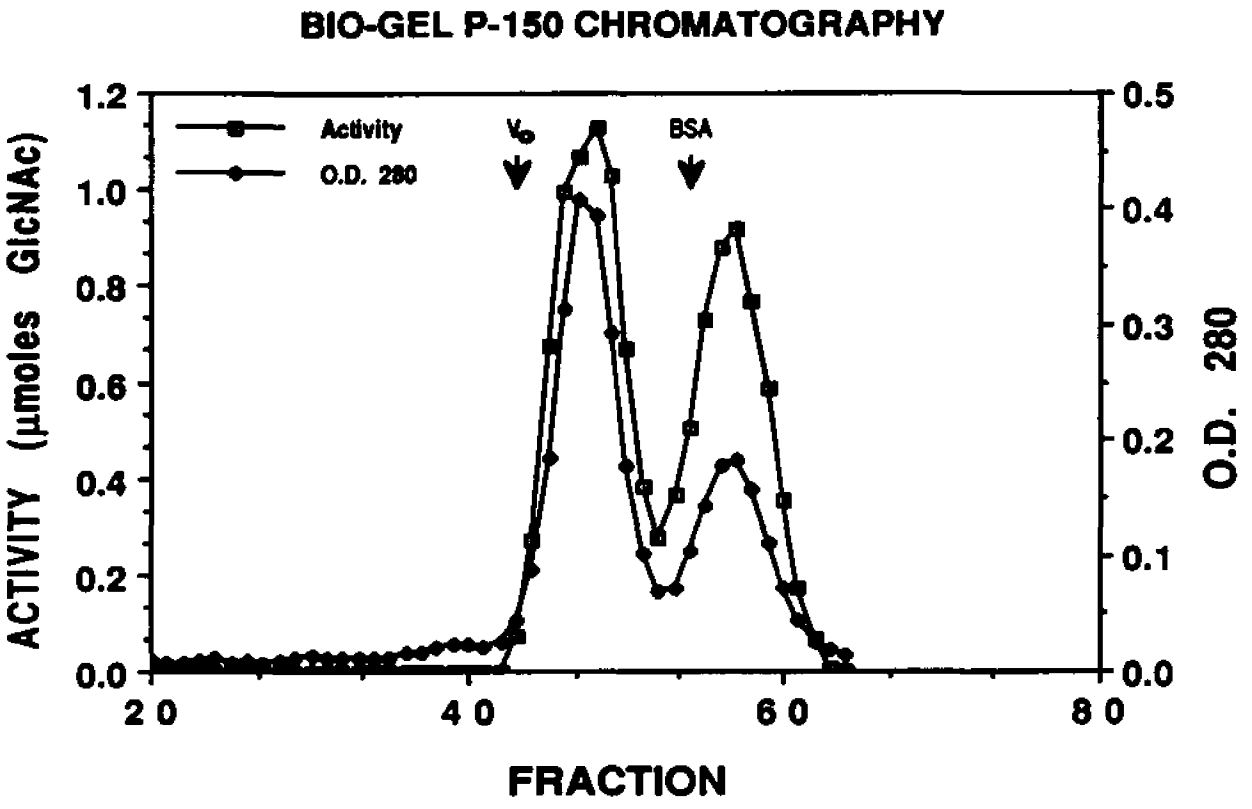


Figure 4.4-A **Chromatography on Bio-Gel P-150 (I).** Fractions eluted from DEAE Bio-Gel A containing chitinase activity (*Figure 4.3*) were chromatographed on Bio-Gel P-150 gel column (see text). The two forms of chitinase exhibit comparable specific activities.

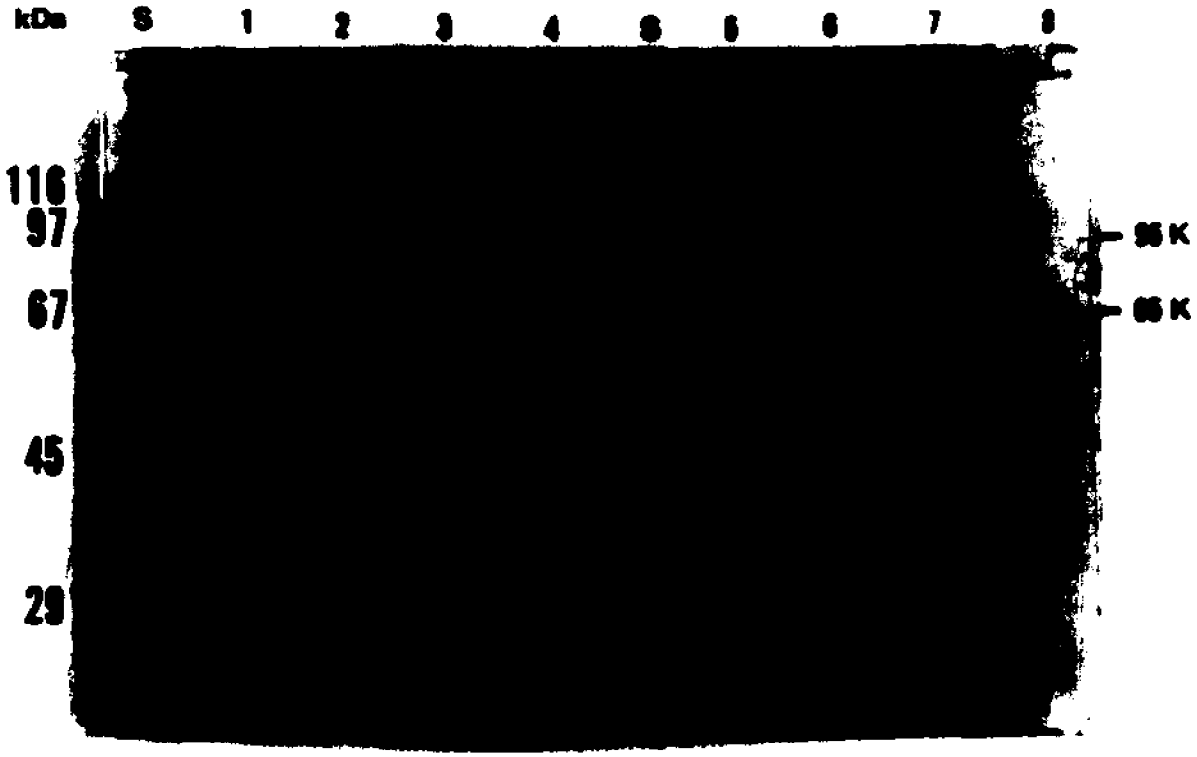


Figure 4.4-B **Chromatography on Bio-Gel P-150 (II).** SDS-polyacrylamide gel electrophoresis (7.5%) on proteins from Bio-Gel P-150 elution (*Fig* 4.4-A). pool I (wells 1-4) and pool II (wells 5-8). Protein load onto fractions 1, 2, 3 and 4 was 10µg, 19µg, 38µg and 77µg; load onto 5, 6, 7 and 8 was 8µg, 16µg, 32µg and 64µg, respectively.

Sequence analysis of pool I and pool II determined that both proteins were blocked at the amino-terminus (Dr. Betty C.-R. Zhu, personal communication).

Some experiments were performed to assess the equivalence between the native and cloned chitinases. Purified chitinase from *E. coli* and *Vibrio parahaemolyticus* were analyzed by electrophoresis on polyacrylamide gel (SDS). The native enzyme appears slightly larger than the cloned chitinase, as the results in *Figure* 4.5 seem to indicate. Similar results were observed on several electrophoresis experiments.

The enzyme was shown to exhibit endo-chitinase activity by detection of fluorescent product upon incubation with 4-methylumbelliferyl-N,N',N''-triacetyl-ß-chitotrioside (Milligan Fossett, personal communication).

4.3 Chitinase characterization

4.3.1 Antichitinase antibodies

The antiserum was affinity-purified by incubation with Sepharose-cloned chitinase gel (see Methods), and tested against chitinase from *V. parahaemolyticus* and *E. coli.* on an Ouchterlony

Figure 4.5 **Gel electrophoresis of native and cloned chitinases.** 7.5 % SDS polyacrylamide gel electrophoresis of purified chitinase from *E. coli* (A) and *V. parahaemolyticus* (B); S= high molecular weight standards.

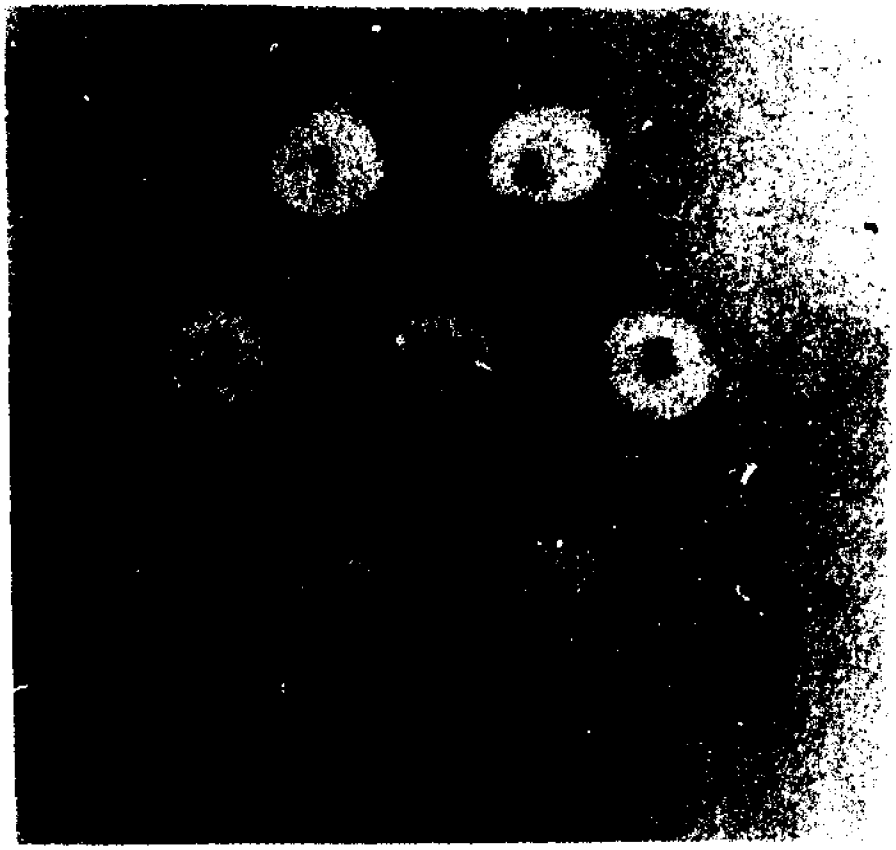


Figure 4.6 **Ouchterlony double diffusion test.** Center well: rabbit polyclonal antichitinase serum (5 µg); wells 1, 2 and 3, purified *Vibrio* chitinase (5µg. 2.5µg, 1.25µg, respectively); wells 4 , 5 and 6, purified chitinase from transformed *E. coli* (5µg, 2.5µg, 1.25µg, respectively).

double-diffusion experiment. Each well was loaded with 50 µl sample. The center well in *Figure* 4.6 contains 5 µg antichitinase antiserum; wells 1-3, serial dilutions of chitinase from *E. coli*, and wells 4-6, serial dilutions of chitinase from*V. parahaemolyticus*. Wells 1 and 4 were loaded with 5 µg of protein. The results indicate that cloned chitinase appears antigenically identical to the native enzyme. Both the native and the cloned chitinase had the same substrate specificity, suggesting that these activities are equivalent.

4.3.2 Chitinase-pH activity profile

The effects of pH on the relative activity of the chitinase was examined for a range in pH from 4 to 10.4. Ten microliter aliquots (0.25 mg/ml) of enzyme were incubated at 50°C for 10 minutes with 190 µl ^{3}H-labeled chitin slurry previously washed with buffer (250 mM) to set the pH.

The buffers used were the following:

Buffer	pH range
Ascorbate	4.0 - 4.4
Acetate	4.8 - 5.2
MES	5.6 - 6.4
PIPES	6.4 - 7.2
TAPSO	7.2 - 8.0

BICINE	8.0 - 8.8
CHES	8.8 - 10.0
CAPS	10.0 - 10.8

The radioactivity of the soluble products was measured as described in Methods. The results are shown on *Figure* 4.7. The enzyme catalyzes the hydrolysis of ^{3}H-chitin for a range of pH between 4.4 and 10, with two optima appearing at pH 6 and 8.8 .

4.3.3 Effect of ionic strength on chitinase activity

Chitinase activity was measured at 50°C, pH 6 and sodiun chloride concentrations ranging from 0 to 6 M. The activity measured in the absence of salt was set arbitrarily to 100 %. The last data point in *Figure* 4.8 corresponds to activity measured under saturation conditions of sodium chloride (approximately 6.1 M). The results indicate that the enzyme is very resistant to ionic strength changes, as it retains more than 25 % of its maximum activity at 4 M sodium chloride.

4.3.4 Effect of temperature on chitinase stability

The effects of temperature on chitinase activity was examined after incubation at different temperatures and pH 6. To determine the stability of the enzyme upon incubation at different temperatures, the enzymatic activity was measured after being preincubated for 30 minutes at the desired temperature. The

results shown on *Figure* 4.9-A indicate that the enzyme exhibits its maximum activity at 50°C.

4.3.5 Temperature dependency of chitinase activity

The activity of the enzyme was studied at temperatures ranging from room temperature (24°C) to 70°C. The enzyme activity was monitored at different times, up to 60 minutes. The results shown on *Figure* 4.9-B indicate the enzyme exhibits the optimal activity at 50°C. No activity was detected at 70°C.

4.3.6 Substrate specificity of Vibrio chitinase

To examine oligomer size specificity, chito-olgosaccharides were prepared by partial acid hydrolysis and purified by gel permeation chromatography (see Materials and Methods). Chitin oligomer concentrates of different degrees of polymerization (D.P.) were incubated with chitinase at pH 6, and the products analyzed on thin-layer chromatography.

Five µl of enzyme (1.6 mg/ml) were incubated for 10 minutes with 95 µl of chitooligomer of D.P. 1-10 (varying from 5 mg/ml to 0.1 mg/ml, respectively). After incubation, 10 µl of the reaction mixture was assayed on thin-layer chromatography silica gel G plates developed with acetonitrile:H_2O (4:1) and visualized by charring. The results on *Figure* 4.10 indicate the chitinase catalyzes the hydrolysis of chitin oligomers with

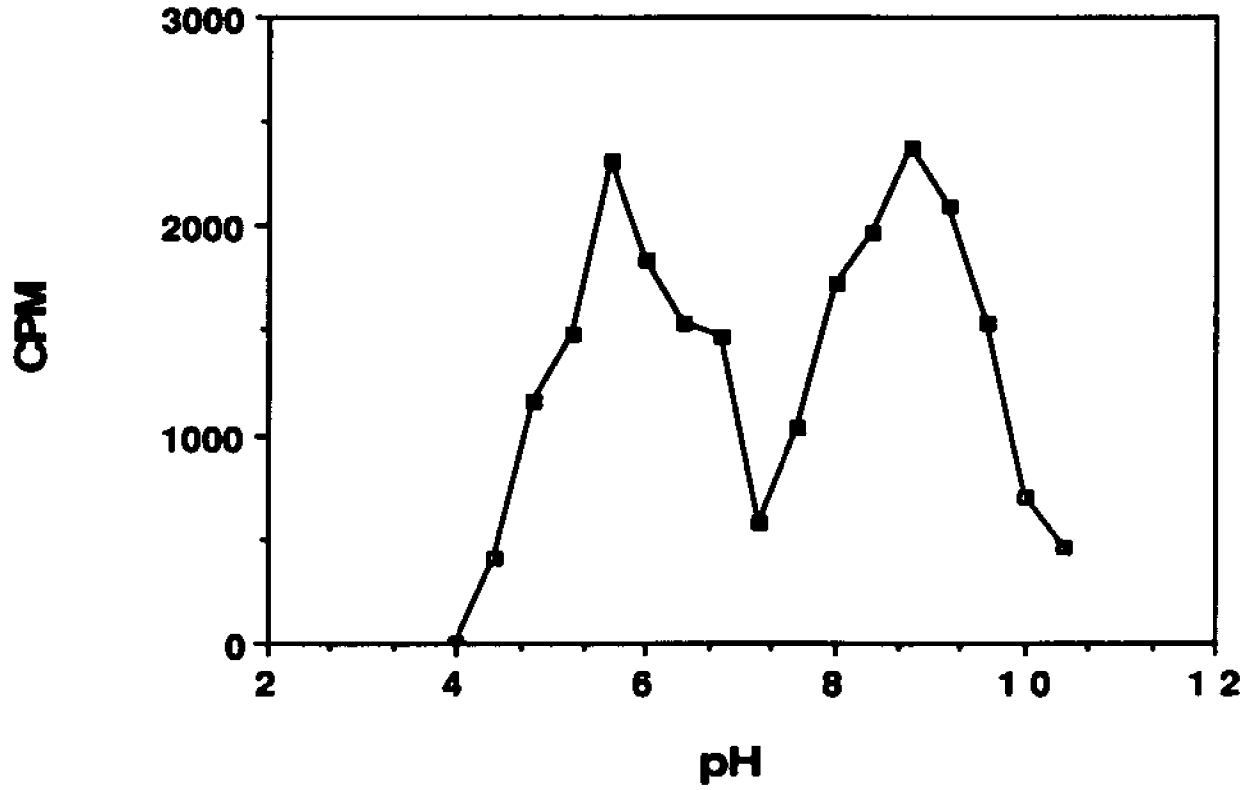


Figure 4.7 **Chitinase activity versus pH.** Ten µl aliquots of chitinase from *Vibrio parahaemolyticus* were incubated for 10 minutes at 50°C with 190 µl of tritium-labeled substrate previously washed with buffer to set the pH. After stopping the reaction with 200 µl TCA (20 %), the mixture was centrifuged and the activity assayed on 200 µl of soluble fraction.

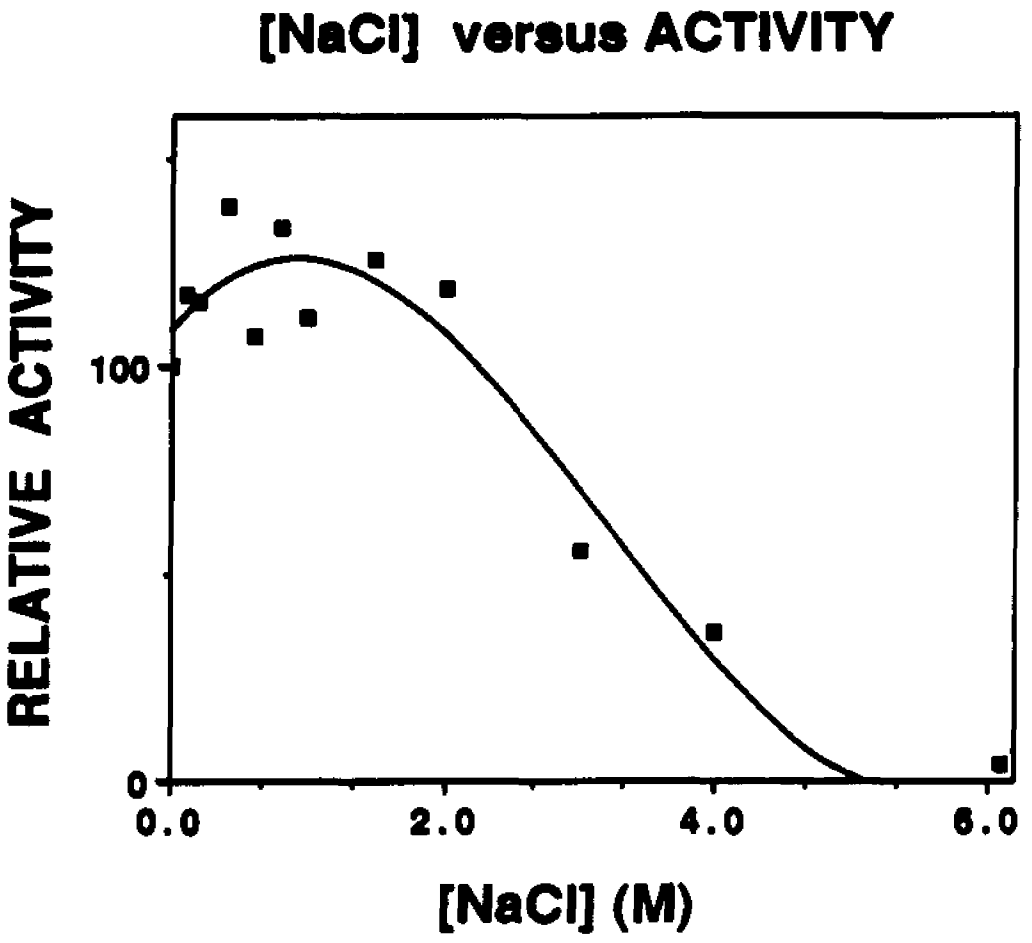


Figure 4.8 **Effect of ionic strength on chitinase activity. Chitinase activity was measured at sodium chloride concentrations ranging from 0 to 6 M, pH 6. The activity in the absence of salt was defined arbitrarily as 100 %. The last data point corresponds to activity measured under saturation conditions.**

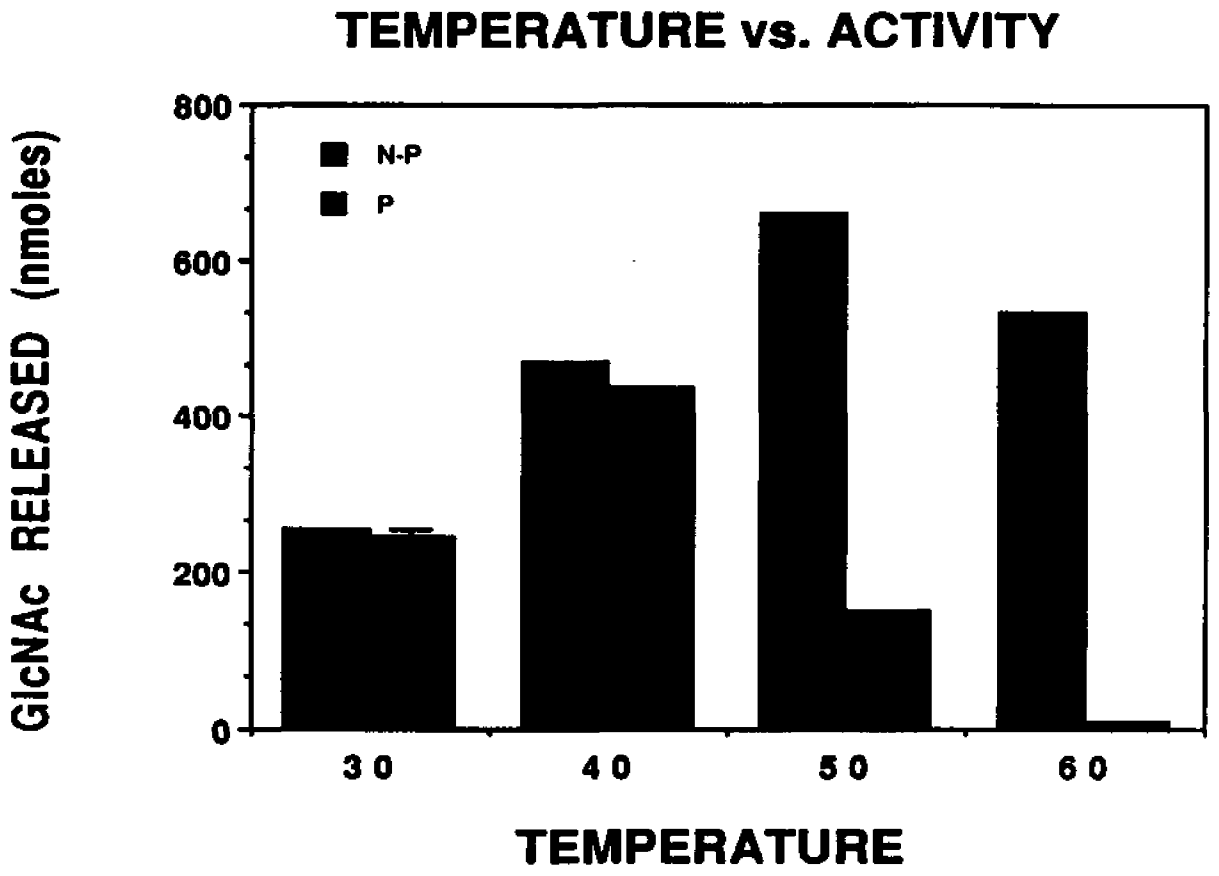


Figure 4.9-A **Temperature versus chitinase activity (I).** Chitinase activity was measured at pH 6 for different temperature from 30°C to 60°C. Stability of the enzyme was examined by measuring activity of samples with or without 30 minutes preincubation (P) at various temperatures.

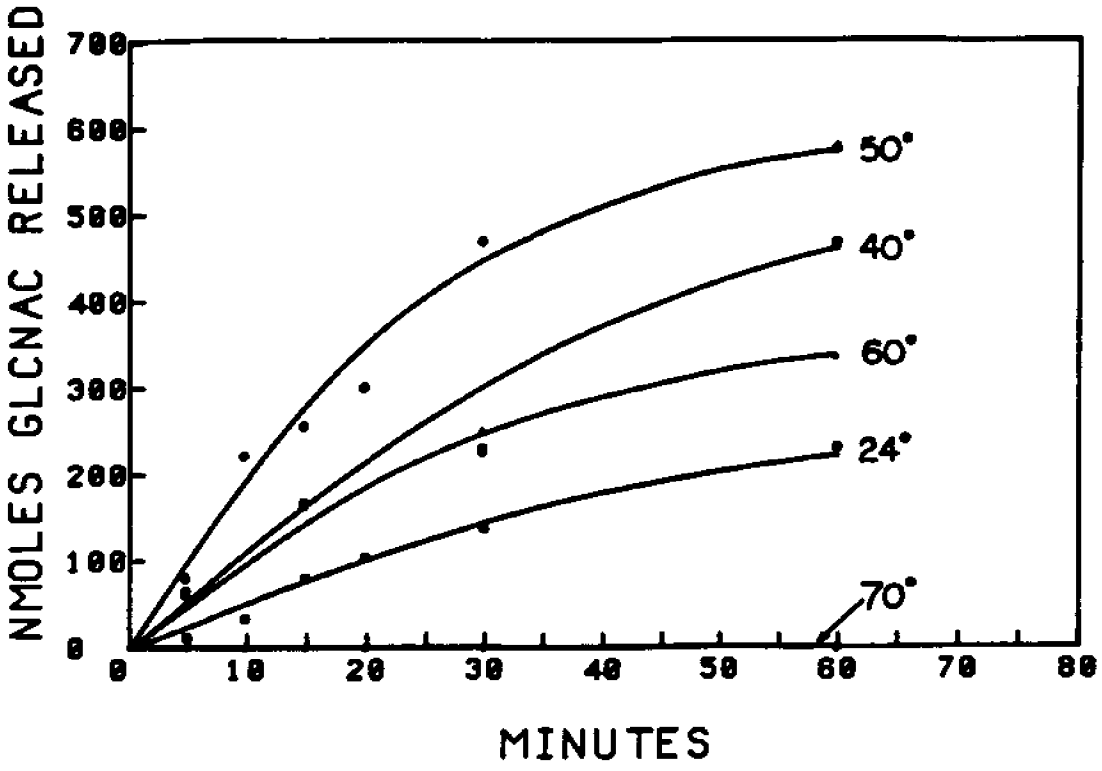


Figure 4.9-B **Temperature versus chitinase activity (II).** Activity was determined at various temperatures for different times up to 60 minutes. The chitinase exhibits maximal activity at 50°C. No activity was detected at 70°C.

degree of polymerization of 3 or higher. The chito-3mer is hydrolyzed slowest, and the enzyme appears to be equally active on substrates larger than the 3-mer.

4.3.7 Transglycosylation activity of chitinase

Hydrolysis of chito-3mer (which gave the lowest hydrolysis rate) was examined at pH 6 and 8.8, the two pH optima of activity. Samples prepared with 64 µl chitotriose (10 mg/ml) and 16 µl buffer (250 mM) were incubated with 20 µl chitinase (0.5 mg/ml) at 50°C for up to 12 hours. The reaction products were analyzed at different times on TLC, and transglycosylation was apparent, especially at pH 8.8 as shown on *Figure* 4.11. Results also show the hydrolysis rate is faster at pH 6 under the experimental conditions.

4.3.8 Induction of chitinase

The addition of chitin to the medium induces the production of high levels of chitinase in *Vibrio parahaemolyticus*, as shown in *Figure* 4.1. For enzyme purification purposes, it seemed important to determine whether the addition to the medium of soluble oligomers of N-acetylglucosamine would produce a similar effect.

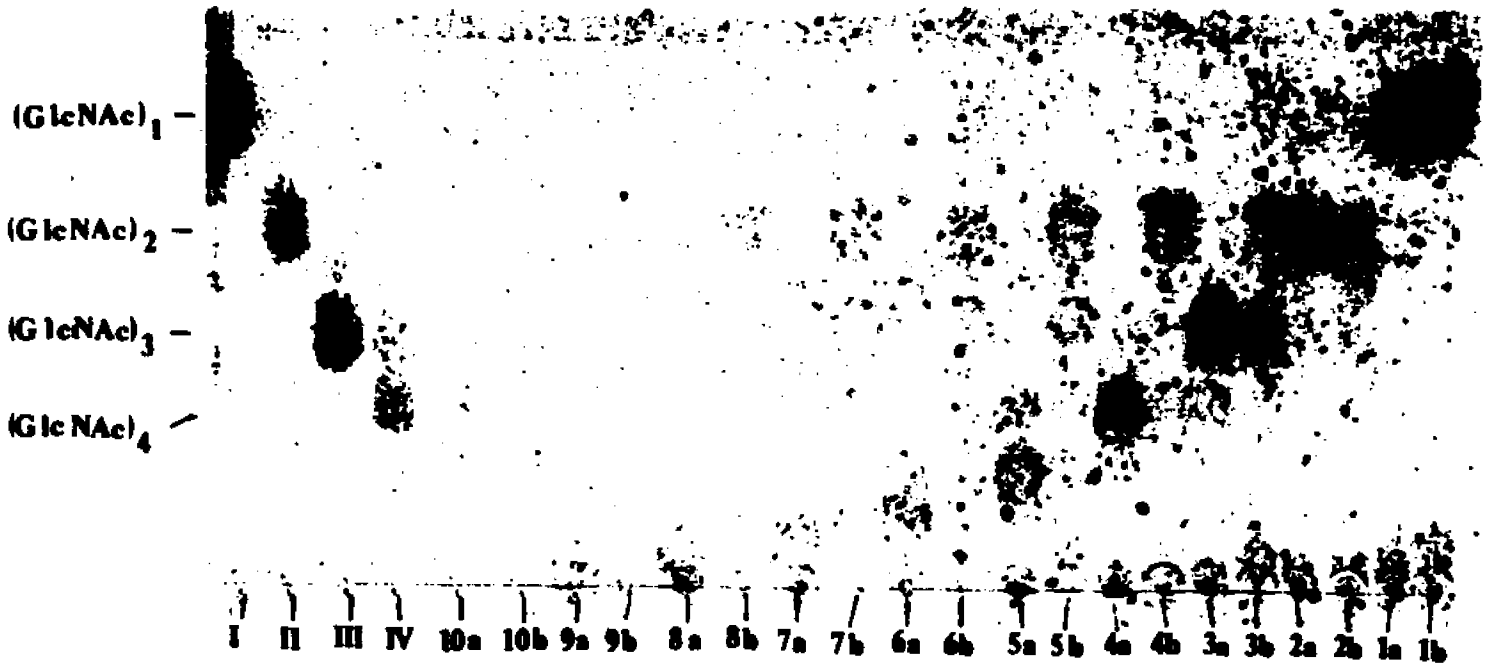


Figure 4.10 **Substrate specificity.** Thin-layer chromatography of chitooligomer concentrates of different degrees of polymerization (prepared as indicated in Materials and Methods) incubated for 12 hours with cloned chitinase at pH 6 and 50°C. Lanes I to IV correspond to standards (2 µg each). The samples were developed on acetonitrile:H_2O (4:1) (see text).

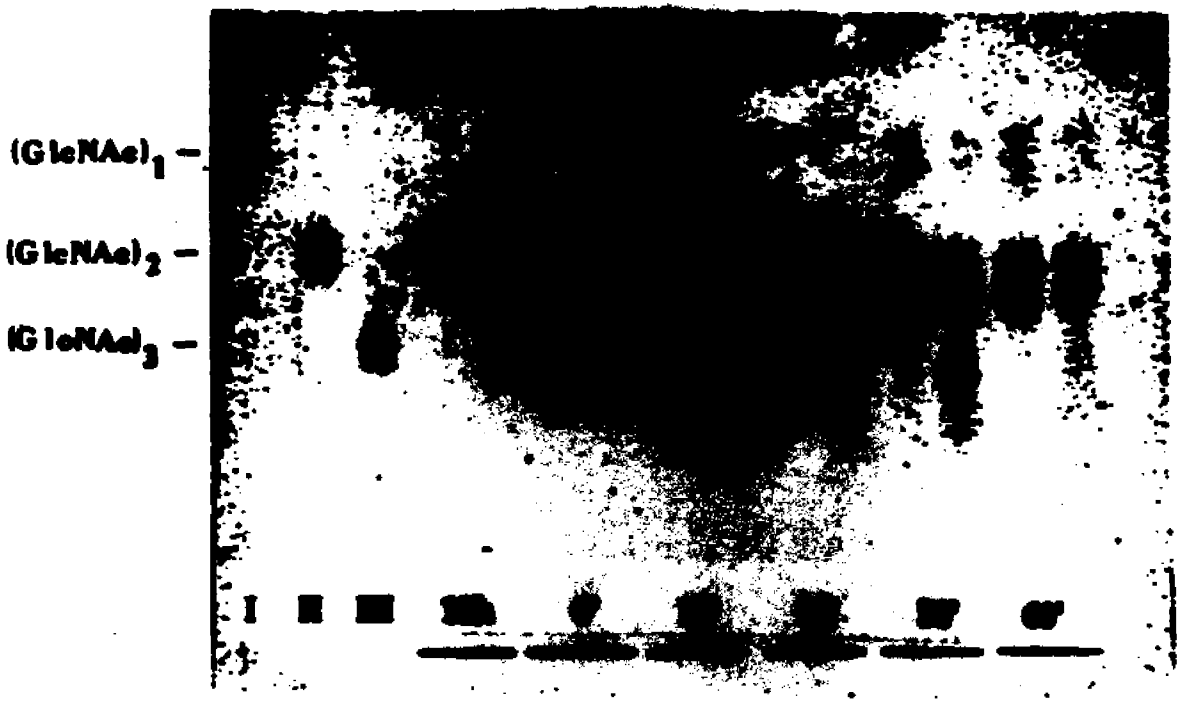


Figure 4.11 **Transglycosylation activity.** Chitinase exhibits transglycosylation activity upon incubation with N,N',N''-triacetylchitotriose. Incubations were carried out at 50°C and the products analyzed by TLC, developed with acetonitrile:H_2O (4:1)(see text).

4.3.8.1 Induction of chitinase by chitooligomers

Vibrio parahaemolyticus (ATCC 27969) was grown in 5 flasks 804 medium supplemented with 1.54 mg/ml of soluble products from chitin hydrolysate. The size of the chitooligosaccharides ranged roughly between mono- and 15-mer. Pentamer was the average average size of the oligosaccharides in this preparation, as determined by thin-layer chromatography, as shown in *Figure* 4.12.

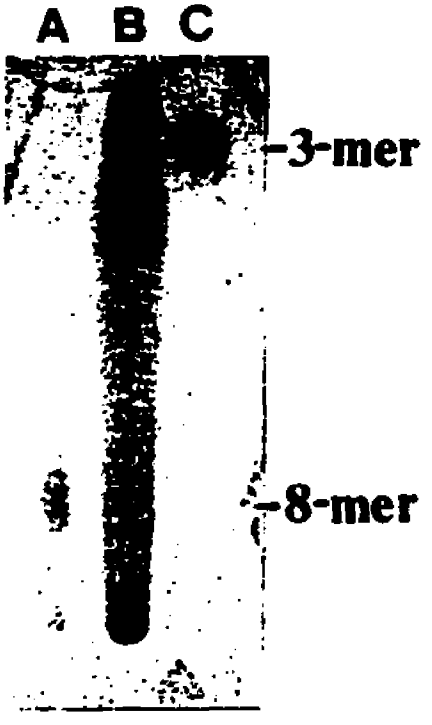


Figure 4.12 Thin-layer chromatography of chitin oligomers. Sample of chitin oligomers used for induction of chitinase in *V. parahaemolyticus* cultures. A= chito-8mer, 0.5 µl; B= oligomer mixture, 1 µl; C= chito-3mer, 1.5 µl. Samples were developed with acetonitrile:H_2O (2.5:1)

Figure 4.13-A **Chitinase induction by chitin oligosaccharides (I). Analysis on 6% SDS-PAGE of proteins released to the growth medium by *V. parahaemolyticus* upon addition of chitin oligosaccharides (1.54 mg/ml).**

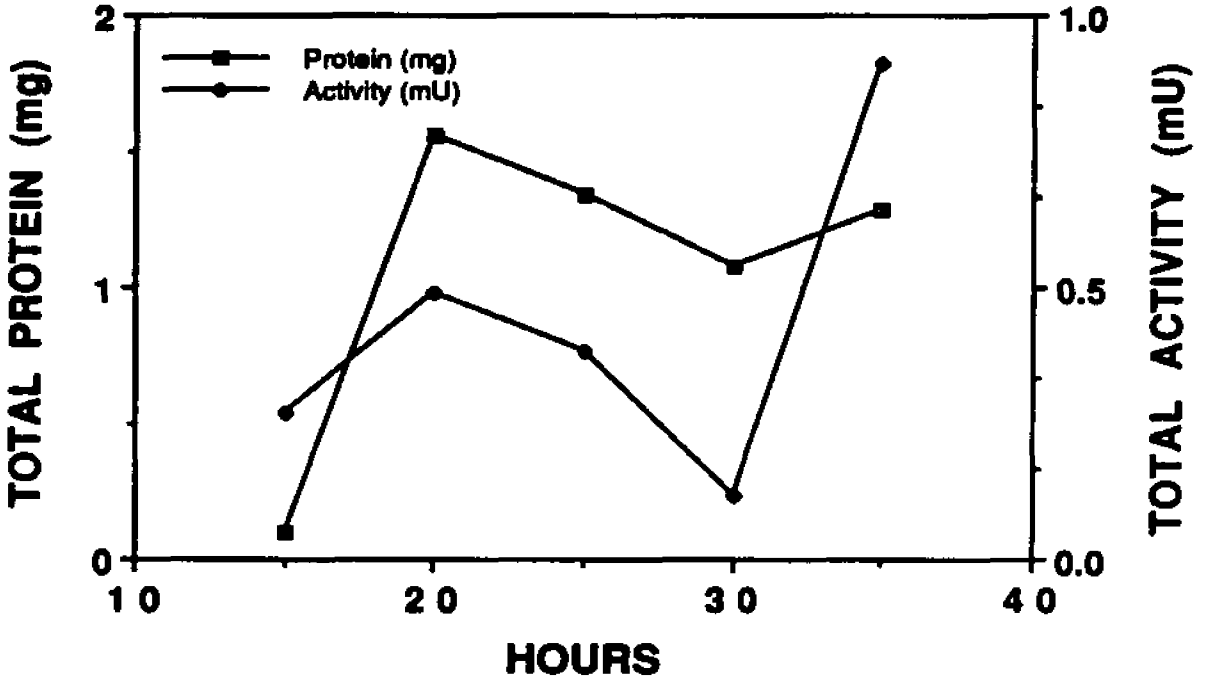
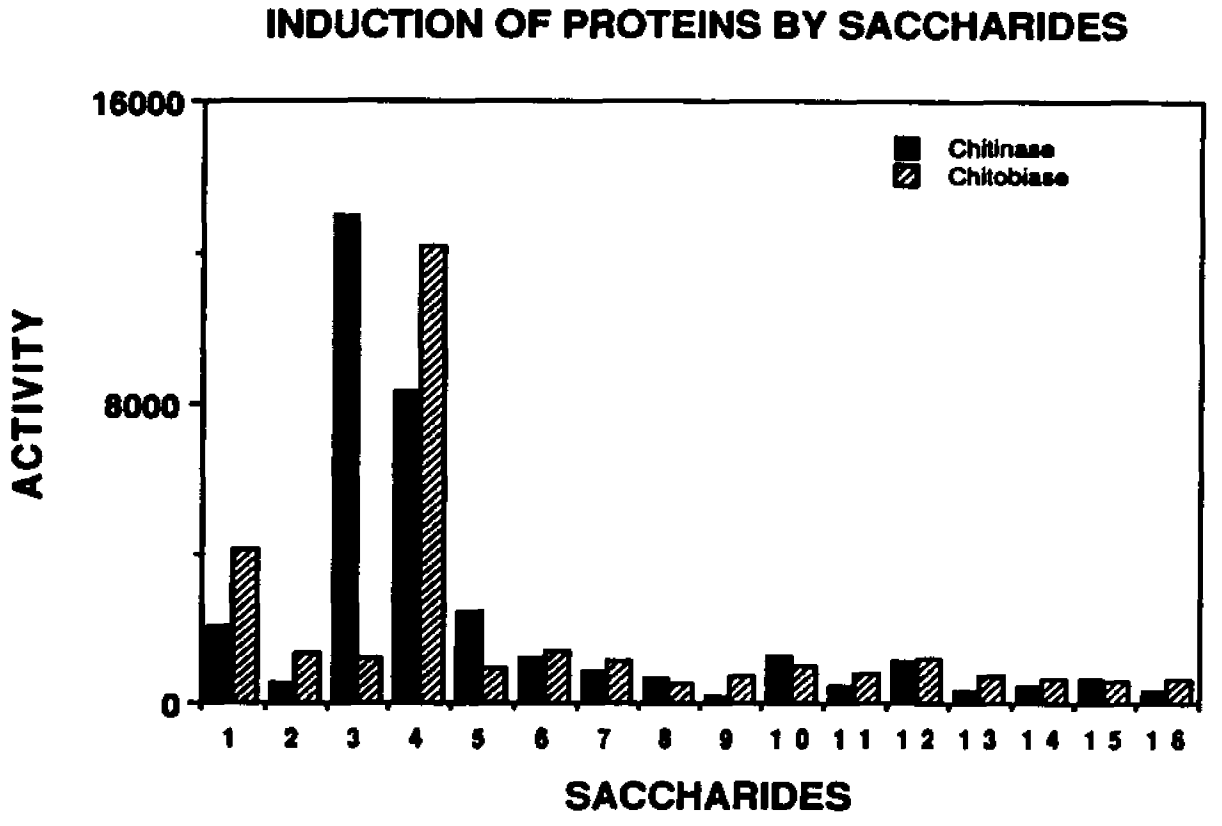


Figure 4.13-B **Chitinase induction by chitin oligosaccharides (II).** Total protein content and chitinase activity in cultures upon addition of chitin oligosaccharides to *V. parahaemolyticus* (see text).

One flask was harvested 15 hours after inoculation and another every 5 hours thereafter. Proteins released to the growth medium were analyzed on 6 % SDS-polyacrylamide gel electrophoresis as shown in *Figure* 4.13-A. The proteins in the culture supernatants were analyzed for chitinase activity and protein content, after ammonium sulfate concentration and dialysis, as shown on *Figure* 4.13-B. The results indicate that *Vibrio parahaemolyticus* secretes chitinase before 15 hours of incubation in the oligomer-supplemented medium. From the gel electrophoresis results, the relative amount of chitinase in the preparations was estimated to be less than 10 % of the total protein.

4.3.8.2 Induction of chitinase by different saccharides

The induction of chitinase was tested on *Vibrio parahaemolyticus* cultures grown on 804 medium and on M9CA (minimal) medium supplemented with 0.57 mg/ml of diverse saccharides, the list of which appears on Appendix A.5. The organism was grown for 10 hours at 37°C in 125 ml Erlenmeyer flasks containing 35 ml of culture medium. After harvesting, the supernatant fractions were analyzed for chitinase and chitobiase activities, and on SDS-PAGE for visual confirmation of the proteins present. The results on Figure 4.14 indicate that other sugars act as inducers of the chitinolytic enzymes in the *Vibrio* organism. The best inducers appear to be di-N-acetyl chitobiose and cellobiose. When chitin is the inducer, the difference in



1.- Cellobiose
2.- Chitin (10h incubation)
3.- Chitin (15h incubation)
4.- Chitobiose
5.- Chitosan
6.- Gentiobiose
7.- GlcNAc
8.- Glucose
9.- Lactose
10.-Maltose
11.-Melibiose
12.-Methyl-ß-glucoside
13.-Methyl-thiogalactoside
14.-Palatinose
15.-Trehalose
16.-Turanose

Figure 4.14 **Chitinase induction by different saccharides.** Chitinase and chitobiase activities of proteins secreted by *Vibrio parahaemolyticus* to growth medium, upon incubation on minimal medium supplemented with diverse saccharides. Cultures were harvested after 10 hours incubation. B = 95K chitinase; C = 65K chitinase; S = high MW standards. Sample 3= 15-hour incubation with chitin.

chitinase production for incubations of 10 and 15 hours seems due to the fact that the enzyme is hydrolyzing the chitin and thus producing more di-N-acetylchitobiose, considered to be a strong inducer of chitinase in *Vibrio harveyi* (Soto-Gil and Zyskind, 1984).

When bacteria were grown on 804 medium alone, the protein was not detected on gel electrophoresis (*Figure* 4.1), although marginal activity was observed as measured by the standard activity assay (not shown).

Chapter 5

Isolation and partial characterization of chitovibrin

5.1 Introduction

The first attempts to purify a protein from *Vibrio parahaemolyticus* that showed chitinase activity included a chitin-binding step. The enzyme, determined to have a molecular mass of 65 kDa, was released by digestion of the substrate during incubation at 37°C. Under these conditions, the protein preparation contained many polypeptides which were believed to be breakdown products of the chitinase.

Chitovibrin came first to our attention while trying to purify the chitinase using a purification protocol that did not include chitin-affinity step after clarifying the culture medium. The release of the proteins bound to chitin substrate was accomplished by incubation in a shaker at 37°C for several hours. In the new procedure, the proteins from the growth medium were precipitated with ammonium sulfate after pelleting and discarding the cells. In addition to a small proportion of the 65K chitinase, the analysis of the proteins in the precipitate revealed a larger concentration of a 95 kDa chitinase, as well as another protein having a molecular mass slightly higher than 120 kDa (later determined to be closer to 134 kDa). In the beginning, the 120 kDa protein was termed "120K" and was considered to be just a

contaminant of the chitinase. When it appeared that 120K exhibited chitin-binding activity, it was renamed chitovibrin.

5.2 Proteins in growth media

Chitinase and chitovibrin could be detected when the growth medium was supplemented with chitin, oligomers of N-acetylglucosamine, and to some minor extent, after the addition of other saccharides (see Chapter 4). As there is no easy way to assay for chitovibrin, it was generally detected as a 120 kDa (120K) protein band on SDS polyacrylamide gels. The first experiments were performed using the wild-type *Vibrio* organism, which was harvested after 3-4 days. The latter induction experiments were done on a *Vibrio* strain (L101) that could be harvested after only 15 hours.

Examination of the proteins in the growth media upon addition of chitin revealed a great variation in the polypeptides secreted by *Vibrio parahaemolyticus*. Supernatant fractions from 6 different *Vibrio* strains were analyzed on 6 % SDS gel electrophoresis as shown in *Figure* 5.1. When plated on chitin-agar, appearence of a halo zone around the bacterial colonies could take from 1 to several days (not shown).

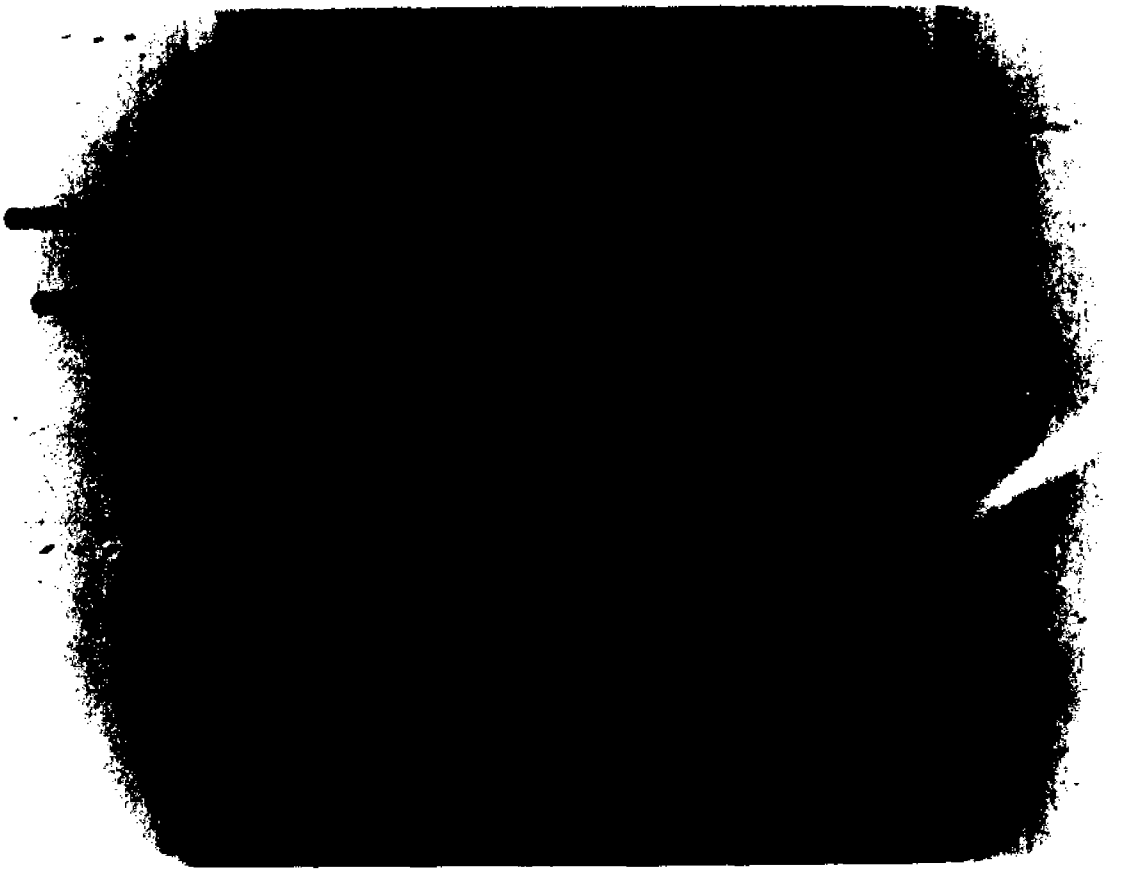


Figure 5.1 **Proteins from different strains of *V. parahaemolyticus*.** Samples of concentrated proteins from supernatant fractions of chitin-induced cultures of *V. parahaemolyticus* isolates.

5.2.1 Localization of chitovibrin in the cell

Wild-type *Vibrio* was grown on 804 medium + chitin. 500ml cultures were harvested after 15, 20, 25, 30 and 35 hours, and 4 different cell fractions were analyzed for the presence of chitovibrin, following a reported procedure (Neu and Heppel, 1965; Nossal and Heppel, 1966). Extracellular (a), hypertonic (b), osmotic shock (c), and cytosolic (d) fractions were analyzed by SDS-PAGE as shown in *Figures* 5.2-A and 5.2-B. The results indicate the presence of a protein band having a similar relative mobility to chitovibrin in the culture supernatant but not in the other fractions obtained. Chitovibrin is observed in the supernatant only after 25 h incubation and seems to be absent in the other fractions. Total protein content from different cellular fractions is shown in *Figure* 5.2-C.

5.3 Gel-permeation chromatography

5.3.1 Bio-Gel P-200

The relative sizes of the 95 kDa chitinase ("95K" chitinase) and the 134 kDa chitovibrin made it difficult to separate them by gel permeation. Separation of the proteins could not be accomplished on Bio-Gel P-150 gel chromatography (not shown). The eluant from this column was concentrated, and a 2.5 ml aliquot was loaded onto a Bio-Gel P-200 gel column (1.5 x 110 cm) and eluted with 50 mM potassium phosphate buffer, pH 6.

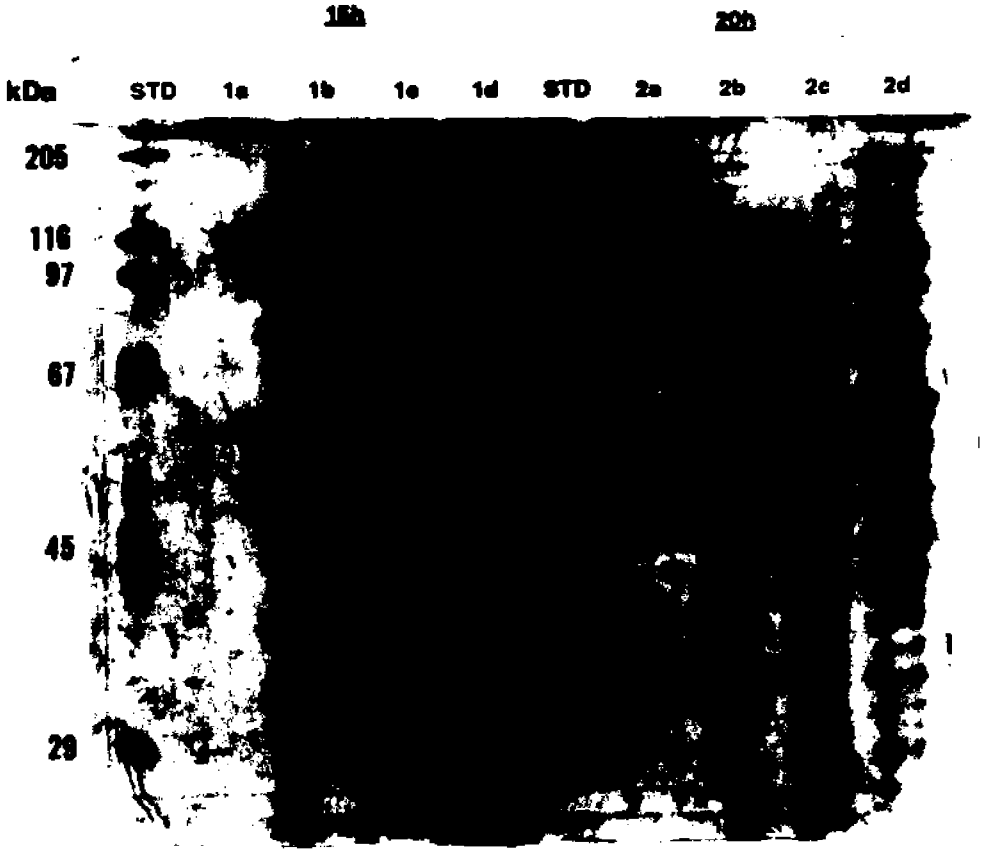


Figure 5.2-A **Localization of chitinase in *Vibrio* cell (I). 15h (1) and 20h (2) samples of different cellular fractions with comparable amounts of proteins were analyzed on 6% SDS-PAGE. a=supernatant fraction; b=hypertonic fraction; c=hypotonic fraction; d=cytosolic fraction (see text).**

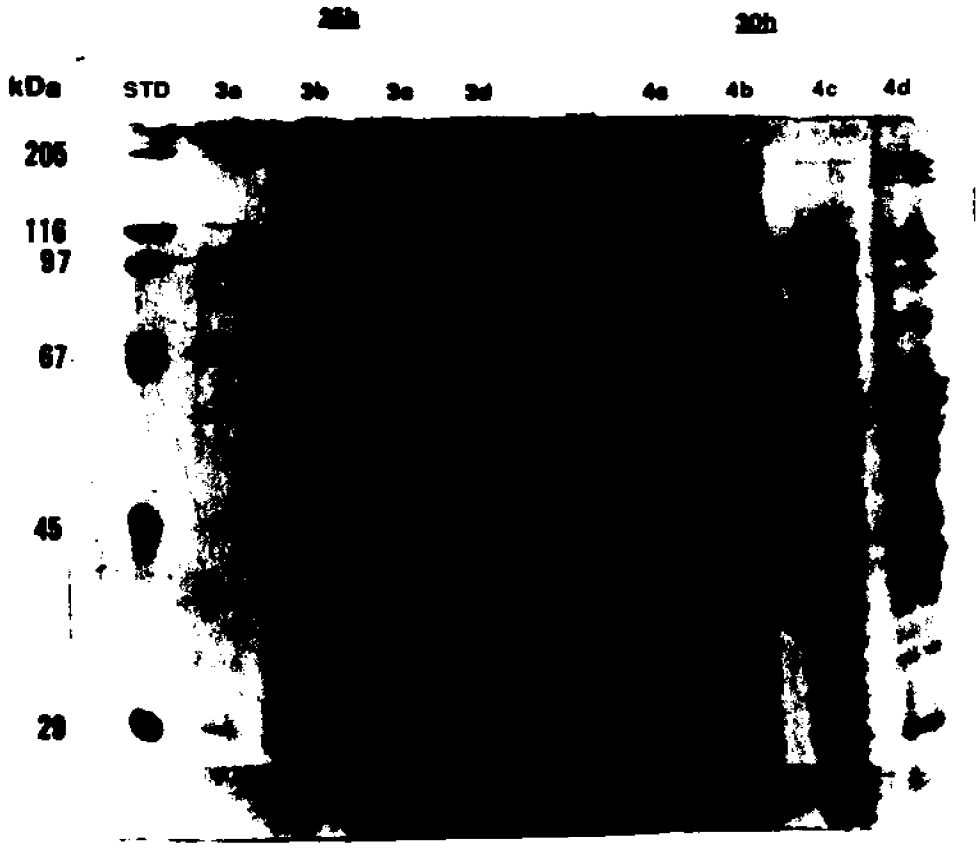


Figure 5.2-B **Localization of chitinase in *Vibrio* cell (II). 25h (3) and 30h (4) samples of different cellular fractions with comparable amounts of proteins were analyzed on 6% SDS-PAGE. a=supernatant fraction; b=hypertonic fraction; c=hypotonic fraction; d=cytosolic fraction (see text).**

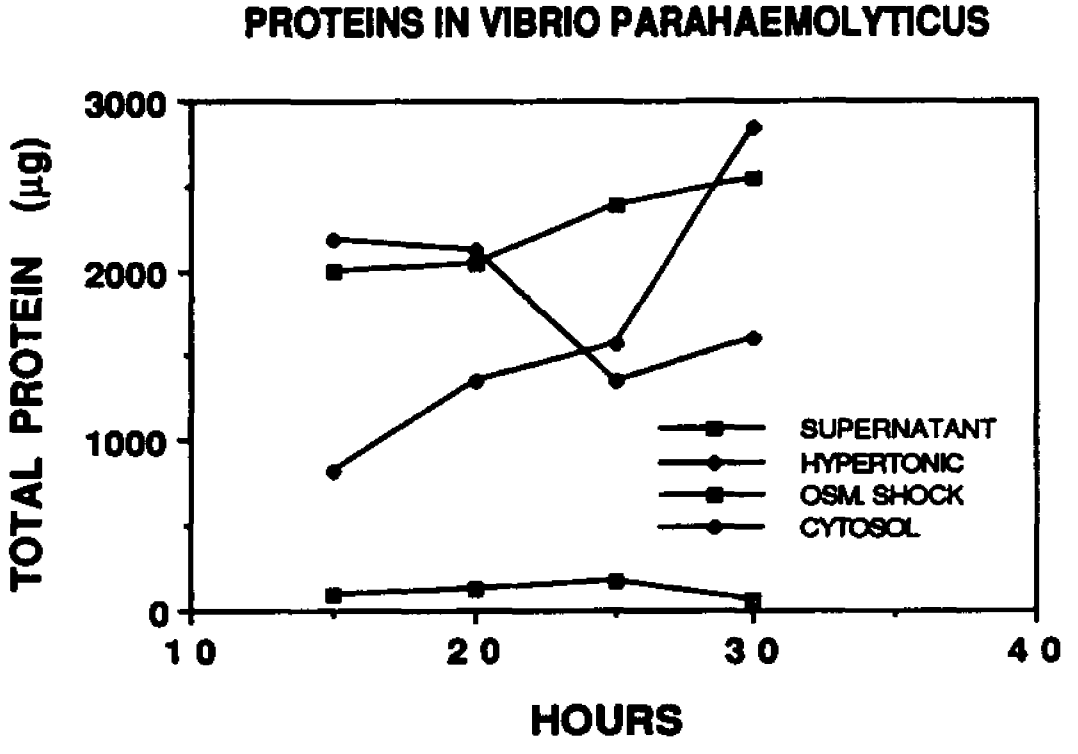


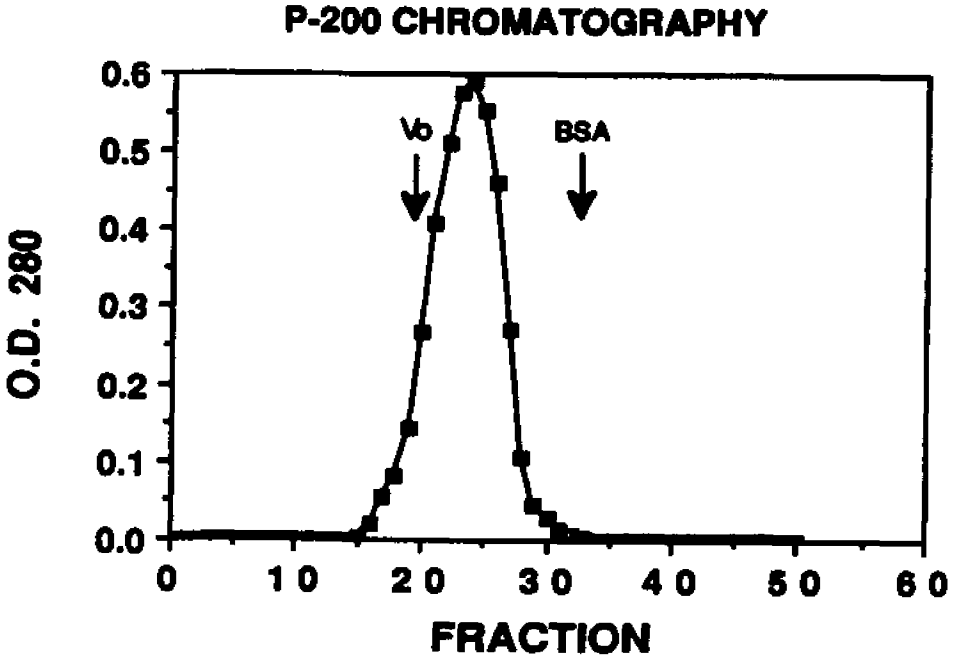
Figure 5.2-C **Localization of chitinase in *Vibrio* cell (III).** Total protein content for different *V. parahaemolyticus* cellular fractions (see text and *Figs. 5.2-A and 5.2-B*).

Fractions of 2.84 ml were collected, as shown in *Figure* 5.3-A. Forty microliter samples from the P-200 elution were electrophoresed on 7.5 % SDS polyacrylamide gel (*Fig* 5.3-B). From P-200 gel chromatography, it could be determined that the proteins eluted from the column with larger apparent sizes than the values obtained through gel electrophoresis.

P-200 gel column chromatography of the proteins was carried out in the presence of 0.3 % octyl-ß-glucoside, a detergent which does not form large clusters and is therefore easier to remove by dyalisis. Fractions of 1.4 ml were collected and assayed by electrophoresis on 6 % SDS-polyacrylamide gel shown in *Figure* 5.4. The results suggest an association between chitovibrin and chitinase which seems to be be removed by the presence of detergent. Fractions 56-59 were later radioiodinated and used in binding studies.

5.3.2 Sepharose-6B chromatography

A crude chitinase sample was chromatographed on a Sepharose-6B gel column (1.5 x 120 cm). Four milliliter fractions were collected and analyzed for protein content, measuring absorbance at 280 nm and on 6 % SDS polyacrylamide gel (*Figure* 5.5). The electrophoresis results revealed proteins having mobilities similar to that of 95K chitinase and 120K protein



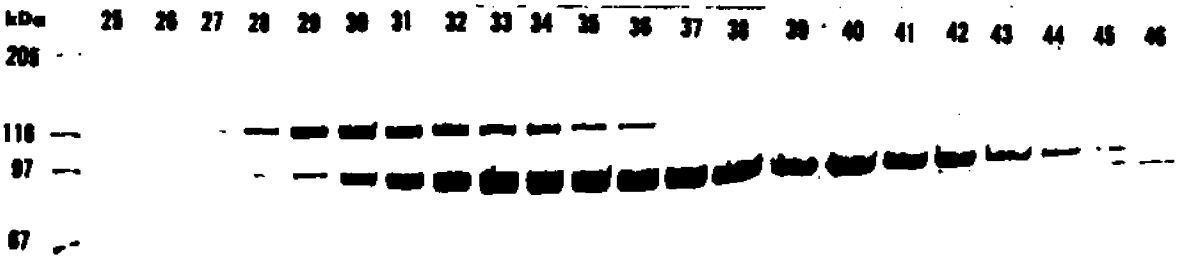


Figure 5.3 **P-200 gel chromatography.** The elution of a protein preparation was observed on 7.5% SDS-polyacrylamide gel electrophoresis and absorbance at 280 nm.

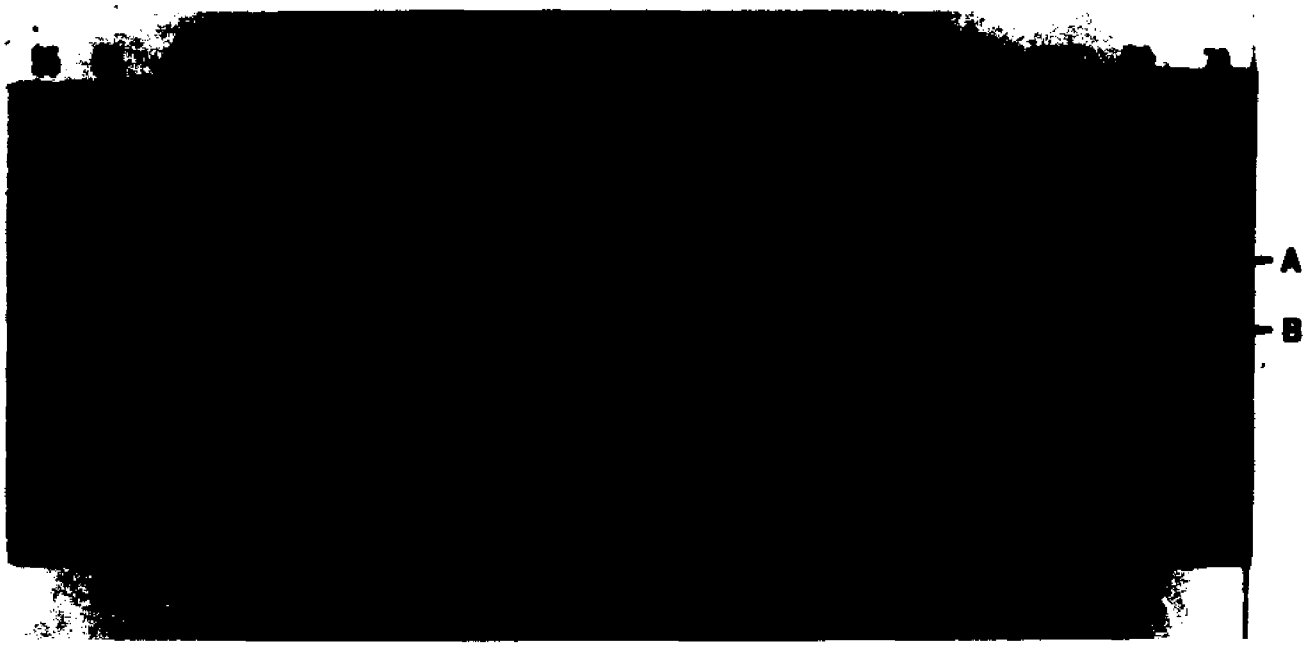


Figure 5.4 **P-200 gel chromatography (+detergent). 6 % SDS-polyacrylamide gel electrophoresis on chitinase concentrate fractions eluted from Bio-Gel P-200 gel column, upon addition of detergent (see text). A= chitovibrin; B= 95K chitinase.**

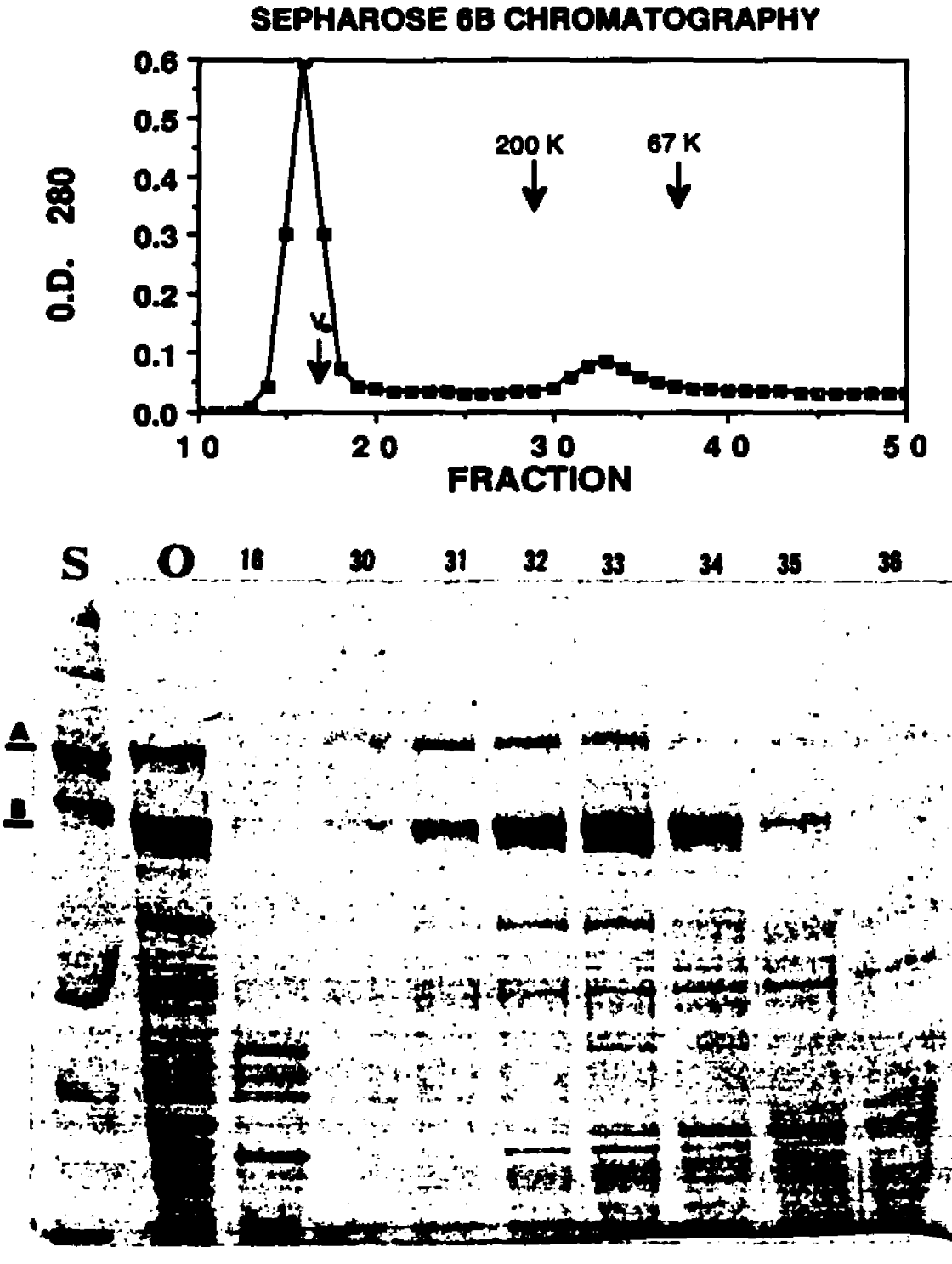


Figure 5.5 **Sepharose-6B chromatography.** Elution of chitinase preparation from Sepharose-6B. I) 6% SDS-polyacrylamide gel; S= high MW standards O=original sample (300 µl); Fraction 16 (300 µl); fractions 30-36 (500 µl) II) Absorbance at 280 nm. A= chitovibrin; B= 95K chitinase.

eluting in the void volume of the colum. The overall pattern of elution did not show a separation of the 2 proteins.

Purification of the chitinase was attempted by allowing it to bind to particulate chitin. The rationale was that the 120K protein could be discarded in the flowthrough and the chitinase recovered by elution with chaotropic agents. The 120K protein was suspected to have chitin-binding properties when trying to purify the 95K chitinase using chromatography on a regenerated chitin column. The preparation consisted mainly of the 95 kDa chitinase and the 120K protein in a ratio of approximately 3:1. Attempts to elute specifically the 120K protein from the column were unsuccessful (results not shown). The following solutions were tried for the elution: 10 mM and 0.5 M p-nitrophenyl-ß-acetylglucosaminide, 0.5 M N-acetylglucosamine, 6 M urea, as well as buffers of increasing pH (from 6 to 9). Urea released the proteins nonspecifically from the column. Suspecting the 120K polypeptide to be a yet uncharacterized chitin-binding protein, it was tentatively named *chitovibrin*.

5.4 Affinity chromatography

In order to purify chitovibrin, as well as to obtain a better characterization of its relationship with chitinase, antibodies were raised against 95K chitinase. Rabbits were inoculated with cloned chitinase, and the antiserum was purified as described by Chua *et al.* (1982) and by Maurer and Callahan (1980). The antibody preparation was then further affinity-purified by chromatography

on a Sepharose-chitinase column, prepared with cloned enzyme. The bound anti-chitinase antibodies were removed from the column, dialyzed and assayed for binding by Western blot analysis with a *Vibrio* protein concentrate (*Figure* 5.6). Proteins transferred to polyvinylidene fluoride membrane (see Methods) were stained with Coomassie Blue (lane A) after incubation with antichitinase antibodies. The bound antibodies were detected by radioiodinated Staph A protein and visualized upon autoradiography (lane B). The results show that chitovibrin does not appear to bind to affinity-purified anti-chitinase antibody. It is important to note that in the Western blot analysis, the results also indicate that the 65K chitinase is recognized only slightly by the anti-chitinase antibody.

Further examination on Ouchterlony double-diffusion gels seemed to support this finding (*Figure* 5.7). Each well was loaded with 50 µl sample (0.1 mg/ml) The center well in *Figure* 4.7 contains 5 µg antichitinase antiserum; wells 1-3, serial dilutions of cloned 95K chitinase from *E. coli*; wells 4-6, serial dilutions of chitovibrin. The results indicate that chitovibrin does not seem to be recognized by antibodies raised against cloned chitinase.

Affinity-purified antibodies were also covalently attached to a Sepharose-4B matrix after activation with cyanogen bromide as described in Methods. A 92 ml sample of protein preparation, containing several polypeptides in addition to chitovibrin and 95K

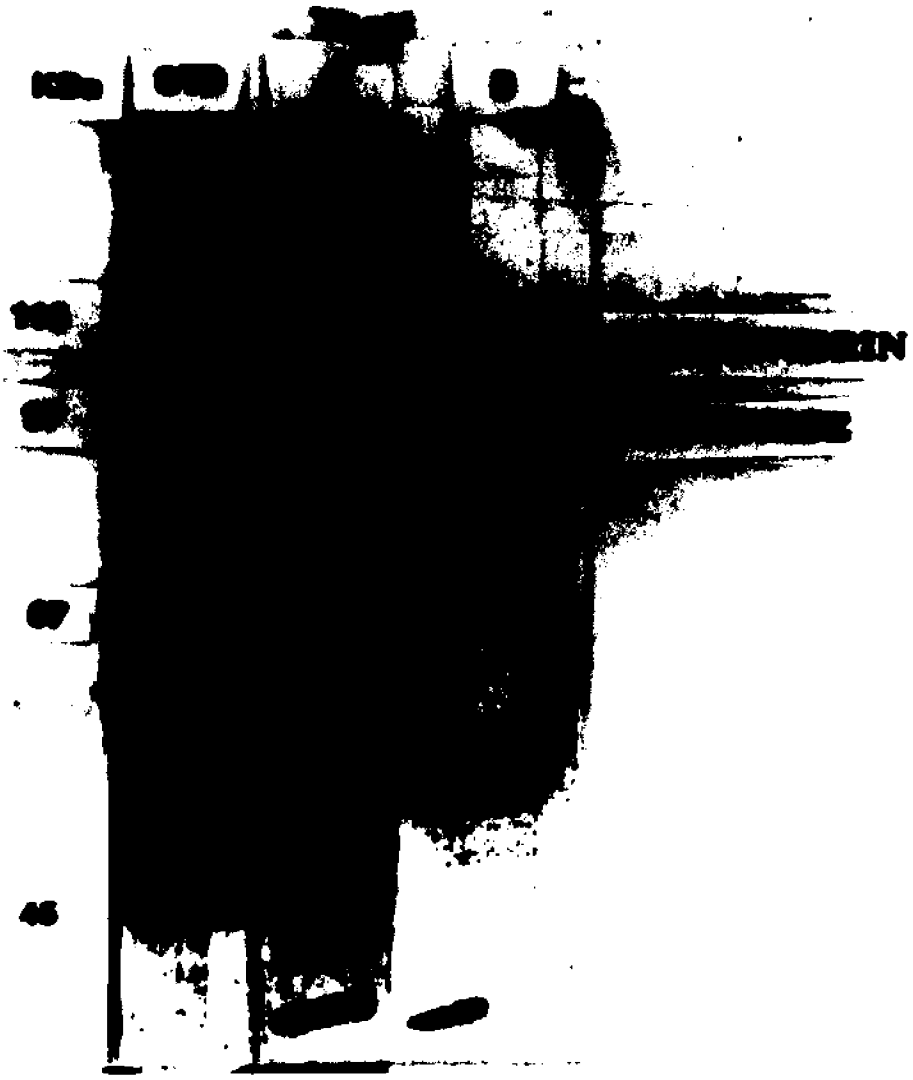


Figure 5.6 **Western-blot analysis. Proteins in crude preparation sample were analyzed for antgenicity against antichitinase antibodies. Lane A, proteins stained with Coomassie Blue; lane B, autoradiography of proteins with bound antibodies.**

Figure 5.7 **Ouchterlony double-diffusion test. Antigenicity of chitovibrin against antichitinase antibodies, examined on Ouchterlony plate. Center well= 5 µg antibodies; wells 1-3, 95K chitinase dilutions; wells 4-6, chitovibrin dilutions (see text).**

chitinase, was passed through a 40 ml column prepared with this affinity gel. Fractions of 6 ml were collected and analyzed by absorbance at 280 nm and gel electrophoresis. Examination of the electrophoresis results in *Figure* 5.8 reveals that most of the chitinase in the original preparation (O) was removed upon incubation with Sepharose-antichitinase antibody gel (lanes 1-4). The proteins appearing on lane 6 are the proteins bound to the affinity gel, which were released with 6M guanidine-hydrochloride and dialyzed extensively. Chitovibrin can be detected in this lane as a minor protein band. The gel-bound sample still retained chitinase activity after dialysis (not shown).

5.5 Hydrophobic interaction chromatography

The use of ion-exchange chromatography to effect purification of chitovibrin was unsuccessful at pH ranging from 4.3 to 9. Later on, when the pI of chitinase and chitovibrin were determined, chromatography on DEAE Bio-Gel A at pH 4 was attempted, but the proteins degraded during incubation (not shown).

The binding and elution of the proteins on hydrophobic gel matrix Phenyl-Sepharose was examined. A 15 milliliter sample of a protein preparation was made 0.8 M NaCl in 10 mM potassium phosphate buffer, pH 7 and then chromatographed on a 3 ml Phenyl-Sepharose CL-4B gel column equilibrated with 0.8 M NaCl in same buffer. The column was washed with the 0.8 M NaCl buffer and then eluted with a 0.8 to 0 M NaCl gradient. The elution profile

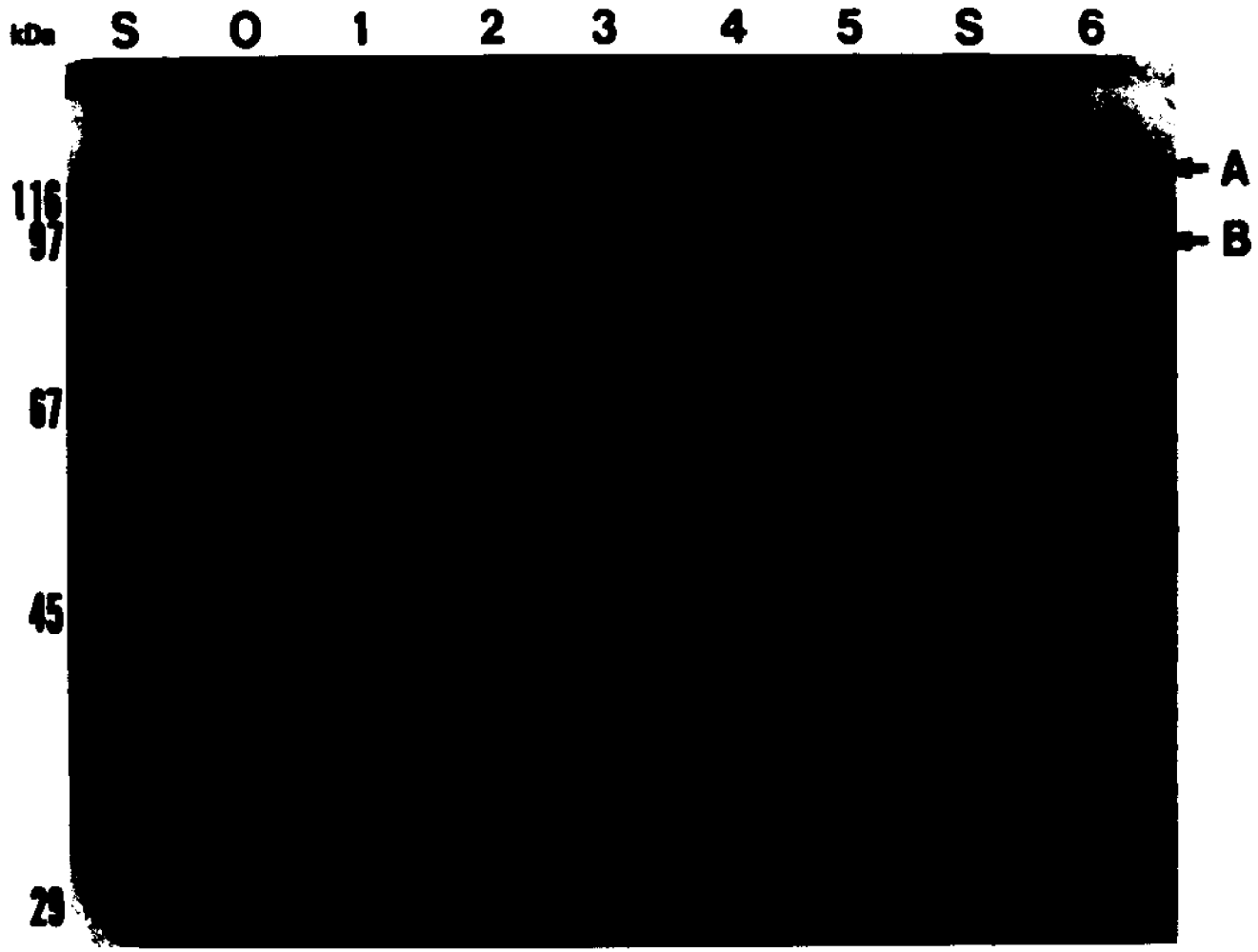


Figure 5.8 **Sepharose-antichitinase antibody column.** Crude sample proteins were analyzed after incubation with antichitinase affinity gel. O= original sample; lanes 1-4, unbound fractions; lane 6, bound fraction. A= chitovibrin; B= 95K chitinase

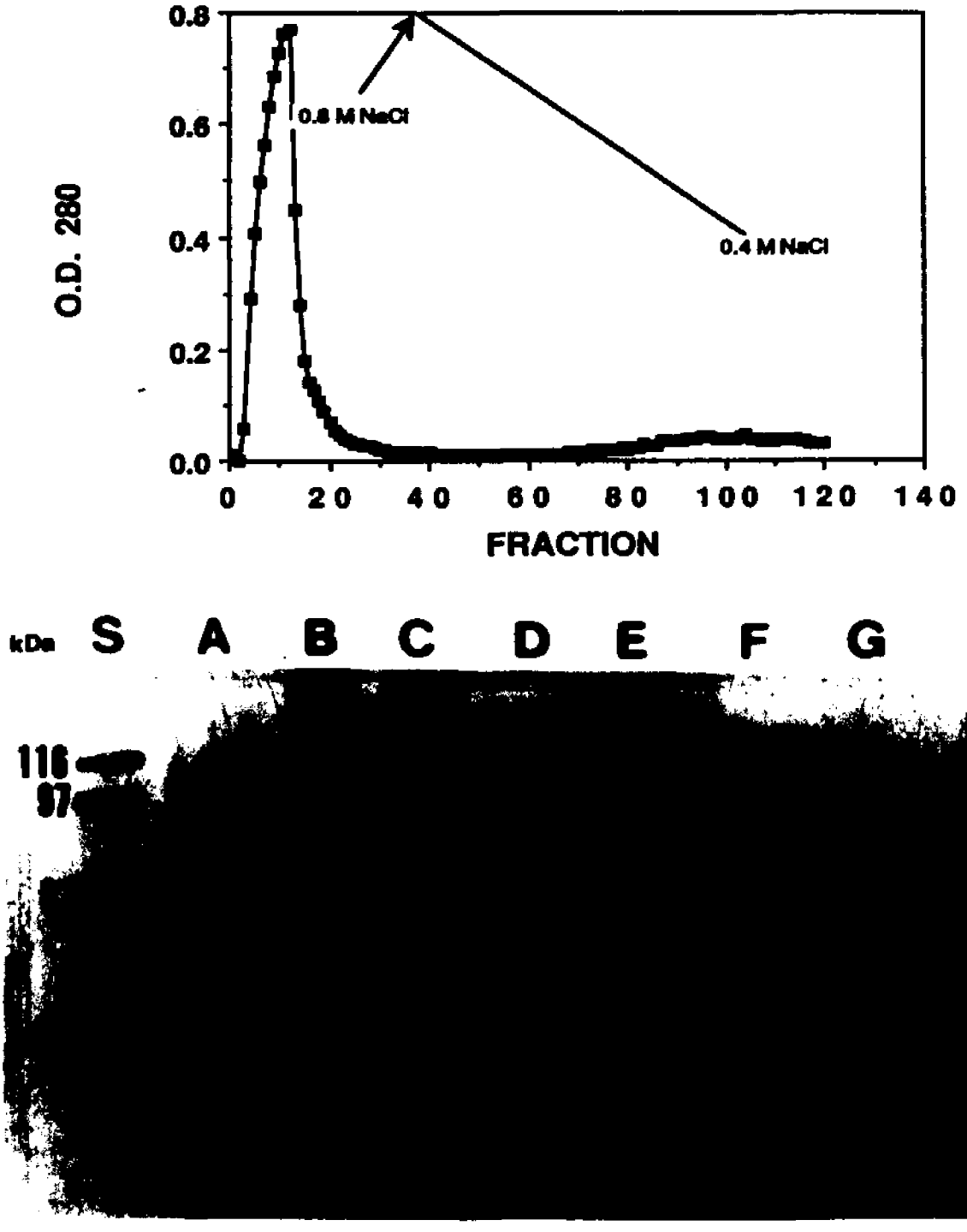


Figure 5.9 **Phenyl-Sepharose chromatography. Sample** chromatographed on Phenyl-Sepharose CL-4B gel column, eluted with buffer at decreasing ionic strength (see text). I) Absorbance at 280 nm; II) 6% SDS-PAGE : fractions 9 (A), 11(B) and 13 (C); pools 85-90 (D), 96-100 (E), 105-108 (F) and 113-116 (G). 1= chitovibrin; 2= 95K chitinase

is shown in *Figure* 5.9. Analysis of the proteins performed on 6 % SDS polyacrylamide gel electrophoresis indicates that chitovibrin, as well as the 65K and 95K forms of the chitinase, bind tightly to this matrix.

5.6 Induction by chitin and chitin oligomers

5.6.1 Induction by chitin

Figure 4.1 shows growth curves and activity profiles of wild-type *Vibrio parahaemolyticus* (ATCC 27969) grown on 804 medium, supplemented (+ch) or not (-ch) with chitin. Proteins examined on 7.5% SDS-polyacrylamide gel electrophoresis, revealed chitovibrin appearing simultaneously with chitinase in culture supernatants.

5.6.2 Induction by chitooligomers

Chitovibrin can be induced in the *Vibrio* organism by oligomers of N-acetylglucosamine, as indicated by the results in *Figure* 4.12. To examine the time during growth when the proteins induced by chitin oligosaccharides are released to the medium, a time-course analysis of the proteins in the culture supernatant was accomplished.

Fifteen different flasks with 35 ml M9CA medium, supplemented with 15 mg of chitin oligosaccharides, were inoculated with *Vibrio parahaemolyticus* and harvested one every hour. Cell density and protein amount in the supernatant fractions

were examined measuring absorbance at 610 nm and 280 nm. Proteins were also analyzed on SDS-polyacrylamide gel electrophoresis, as shown in *Figure* 5.10. The results suggest that chitovibrin is released to the medium as early as 4 hours after inoculation, under the experimental conditions.

5.6.3 Induction by different saccharides

The induction of the proteins was tested on 10-hour *V. parahaemolyticus* cultures grown on 804 medium and on M9CA (minimal) medium supplemented with 0.57 mg/ml of diverse saccharides, as described in Chapter 4. The protein profiles appear in *Figure* 5.11. The list of saccharides supplemented to the media, as well as the presence of protein bands showing similar mobility as chitovibrin is indicated in Table 5.1. A different induction experiment was carried out for 15-hour cultures, with supplemented saccharides at a concentration of 1.25 mg/ml. After harvesting, the proteins in the supernatant fractions were analyzed for the chitovibrin band on SDS-PAGE as shown on *Figure* 5.12.

5.7 Chitin column chromatography

5.7.1 Unlabeled chitovibrin

To further (apart of results in *Fig.* 4.2) examine binding specificity of chitovibrin to a chitin substrate, differential elution of the chitin-bound proteins was studied. As mentioned before

Figure 5.10 **Time-course analysis of induction by chitin oligomers.** Growth upon induction with chitin oligomers was followed every hour analyzing the proteins secreted on 6% SDS-PAGE. A= chitovibrin; B= 95K chitinase.

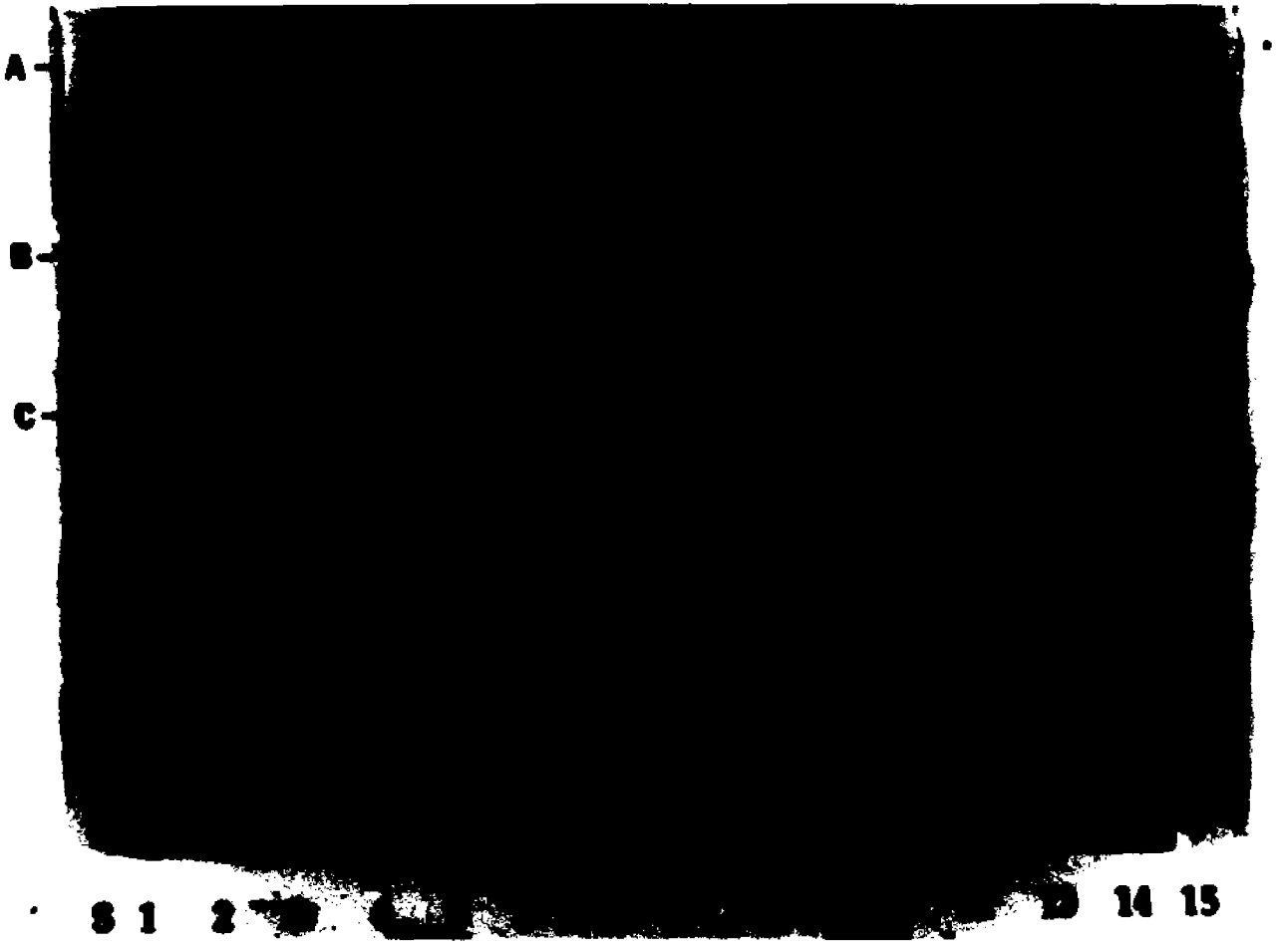
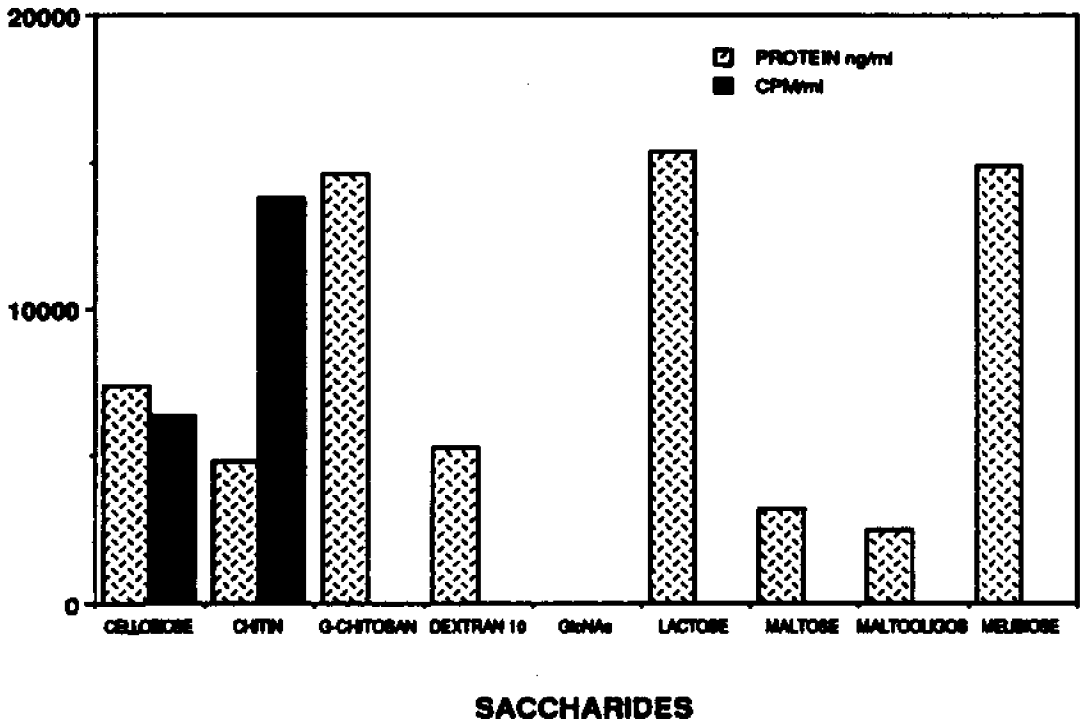


Figure 5.11 **Induction by different saccharides (I). Analysis on 6% SDS-PAGE of proteins secreted by *Vibrio parahaemolyticus* to growth medium, upon incubation on minimal medium supplemented with diverse saccharides (on Table 5.1). Cultures were harvested after 10 hours incubation. A= chitovibrin; B = 95K chitinase; C = 65K cchitinase; S = high MW standards**



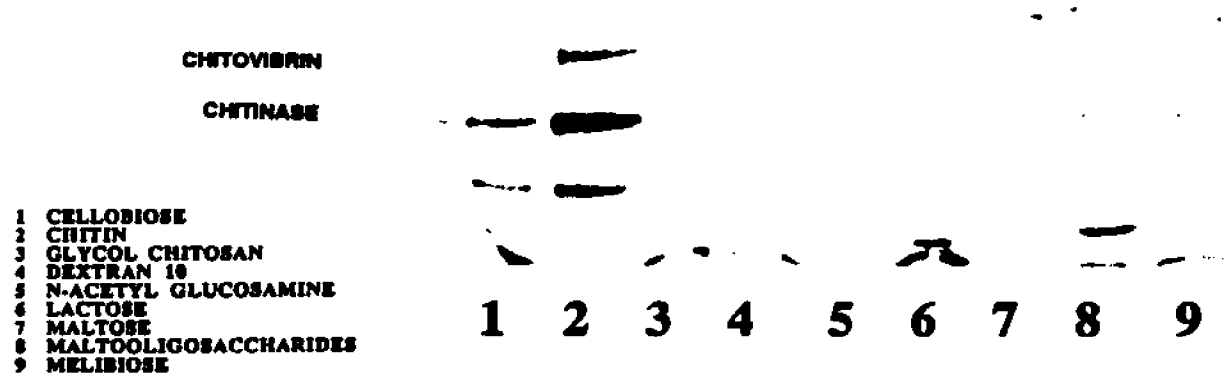


Figure 5.12 **Induction by different saccharides (II). Analysis on 6% SDS-PAGE of proteins secreted by *Vibrio parahaemolyticus* to growth medium, upon 15h incubation on minimal medium supplemented with diverse saccharides. Protein content and activity profiles appear in the upper panel.**

Table 5.1

Induction of chitovibrin by different saccharides

Saccharide	Chitovibrin
1.- Cellobiose	+
2.- Chitin (10 h)	-
3.- Chitobiose	+++
4.- Chitosan	-
5.- Gentiobiose	++
6.- N-acetylglucosamine	++
7.- Glucose	-
8.- Lactose	*
9.- Maltose	*
10.- Melibiose	*
11.- Methyl-ß-glucoside	*
12.- Methyl-thiogalactoside	*
13.- Palatinose	*
14.- Trehalose	-
15.- Turanose	+

+ band present; - band absent; * approximate-size band present

(*Fig.* 5.8), one method to remove the bound chitinase and chitovibrin is to wash the column with 6 M guanidine hydrochloride (G-HCl). The relative strength of the binding to chitin by chitinase and chitovibrin was observed by eluting with increasing concentrations of guanidine-HCl. A 10 ml sample of crude preparation, centrifuged to remove some floculation that had formed in the solution while stored at -20°C, was passed through a 3 ml column of regenerated chitin. The column was subsequently washed with 8 ml of PBS and then eluted with 1 ml batches of increasing concentrations, ranging from 0.12 M to 6 M guanidine-hydrochloride. The fractions obtained were dialyzed against H_2O. Chitinase activity and protein concentration appear in *Figure* 5.13-A. Proteins analyzed on 6% SDS-PAGE, shown in *Figure* 5.13-B, indicate that chitinase is released at lower concentrations of guanidine-hydrochloride then chitovibrin. An 80-85 kDa protein that coelutes with chitovibrin may be a breakdown product of this protein, as the binding to the chitin column seems to be equally strong for both protein moieties, and the protein band is not observed in the original sample (O).

5.7.2 Labeled chitovibrin on chitin column

A 1 ml column of regenerated chitin was used to chromatographed a chitovibrin-enriched sample that had been radiolabeled with ^{125}I. The column was eluted with different solutions, as shown in *Figure* 5. 14-A. The autoradiogram of the

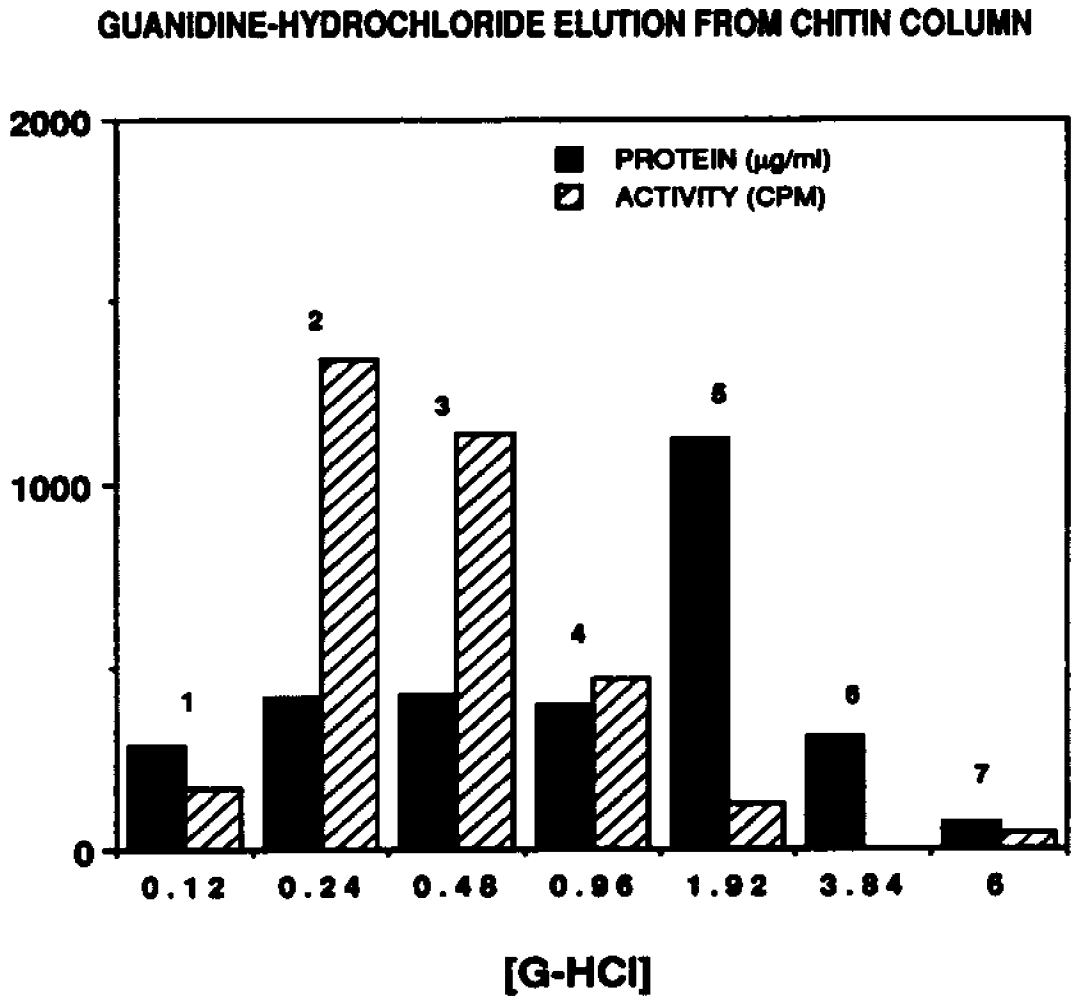


Figure 5.13-A **Chitin column: guanidine-hydrochloride elution (I). Protein content and chitinase activity of different fractions eluted from chitin column with batch-gradient of guanidine-hydrochloride.**

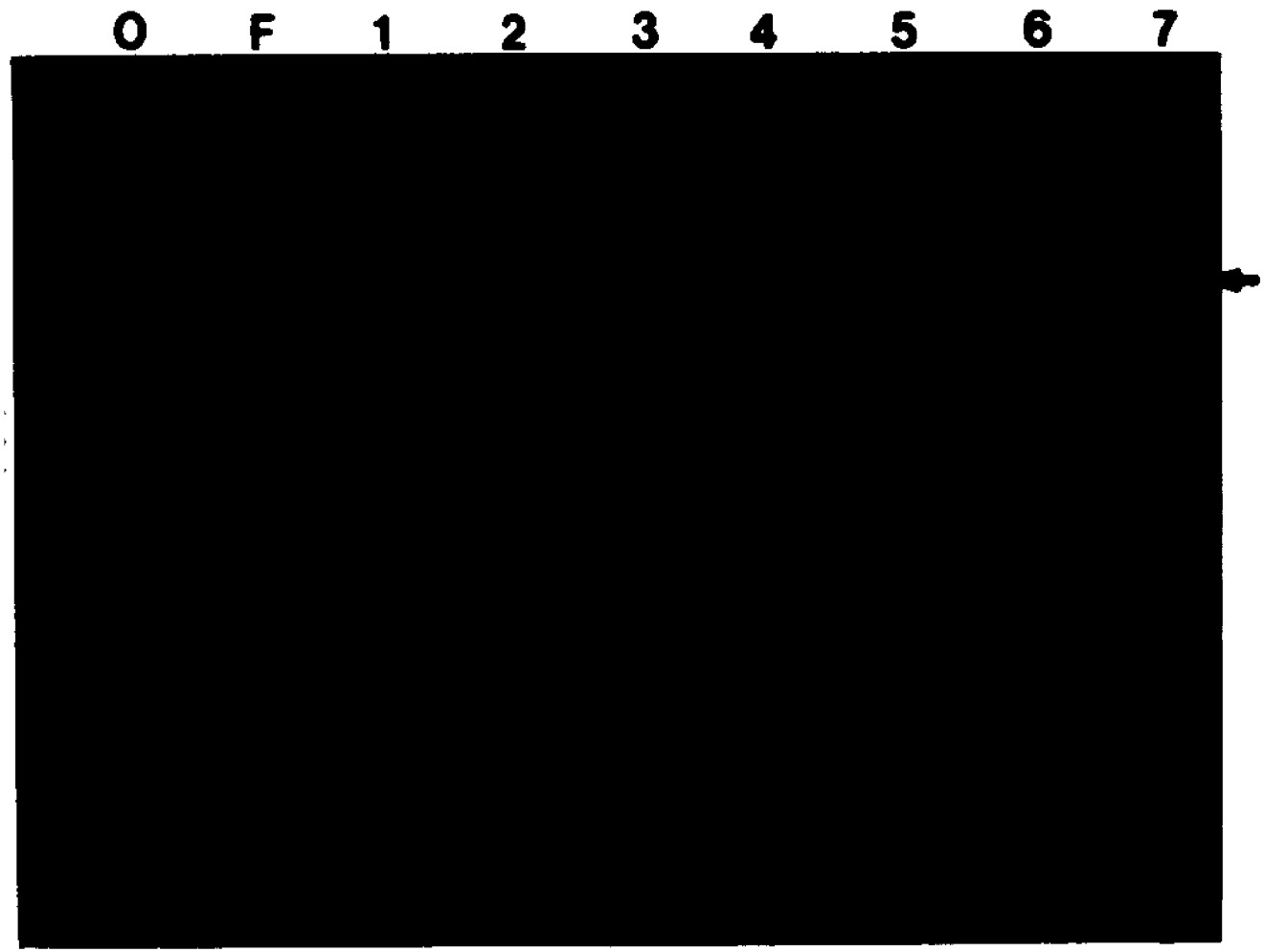


Figure 5.13-B **Chitin column: guanidine-hydrochloride elution** (II). Unlabeled protein concentrate eluted from chitin column with batch-wise gradient of guanidine-chloride concentrations. The arrow points to the chitovibrin protein band on the 6% SDS-polyacrylamide gel. The fraction numbers correspond to batch-elution in *Figure* 5.13-A. O= original sample; F= flowthrough.

eluted fractions presented on *Figure* 5.14-B suggests a specific binding of chitovibrin to the chitin matrix. This figure also shows that chitovibrin may be eluted from the column with concentrated chitin oligosaccharides (approx. 30 mg/ml).

5.8 Affinity to chitin oligomers

To assess the binding affinity of chitovibrin to chitin oligomer substrates, binding assays of chitovibrin with immobilized chitin oligomers were performed. Chitin oligomers were also used to prepare neoglycolipids, and assayed for specificity of binding to chitovibrin on 96-well plates.

5.8.1 Radiolabeled chitovibrin

A column prepared with 1 ml of Sepharose-chitooligomer affinity gel was used to examine the binding of chitovibrin to chitin oligomers. A sample of ^{125}I-labeled protein was loaded onto the column. Proteins were eluted with 2N NaCl, before eluting with guanidine-hydrochloride. Eluted fractions were analyzed by autoradiography. The results, shown in *Figure* 5.15, suggest a specifically strong binding of chitovibrin to the chitin matrix.

In a related experiment, and in order to examine the low binding of the radiolabeled chitovibrin to chitin, as compared with unlabeled samples, fraction 2 from the experiment in *Figure* 5.15 was re-chromatographed on the same chitin column after equilibrating back with PBS buffer. The elution is presented in

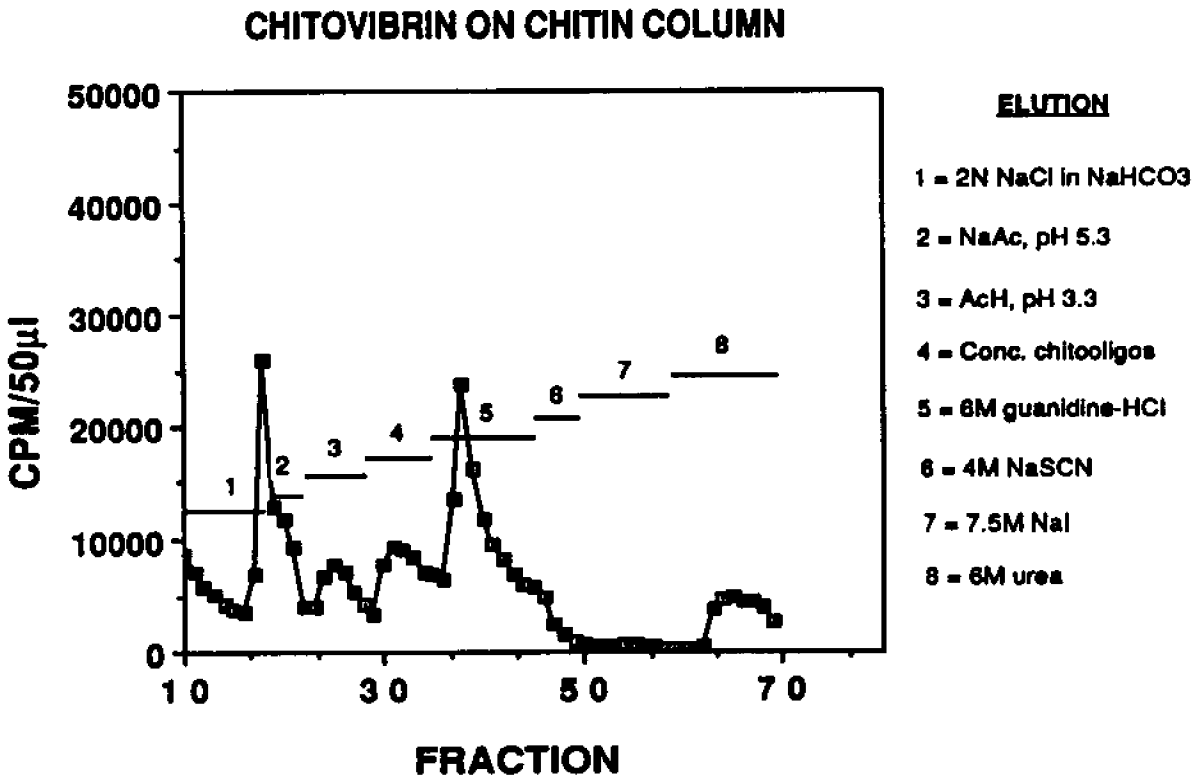


Figure 5.14-A **Chitin column: elution of radiolabeled proteins (I).** The elution from chitin column of radiolabeled chitovibrin concentrate with different solutions.

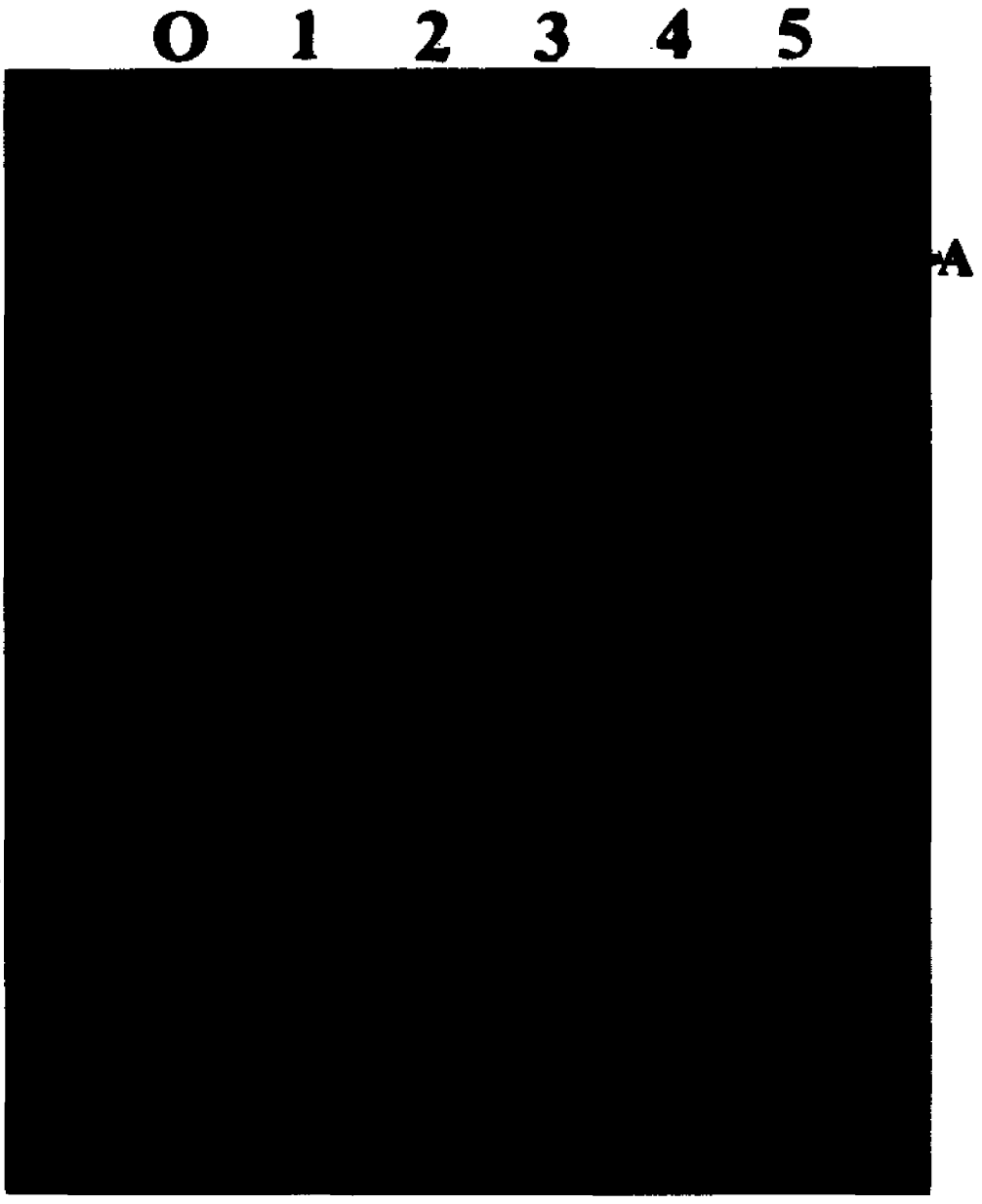


Figure 5.14-B **Chitin column: elution of radiolabeled proteins (II).** Autoradiography of proteins eluted from chitin column with different solutions (see text). A= chitovibrin.

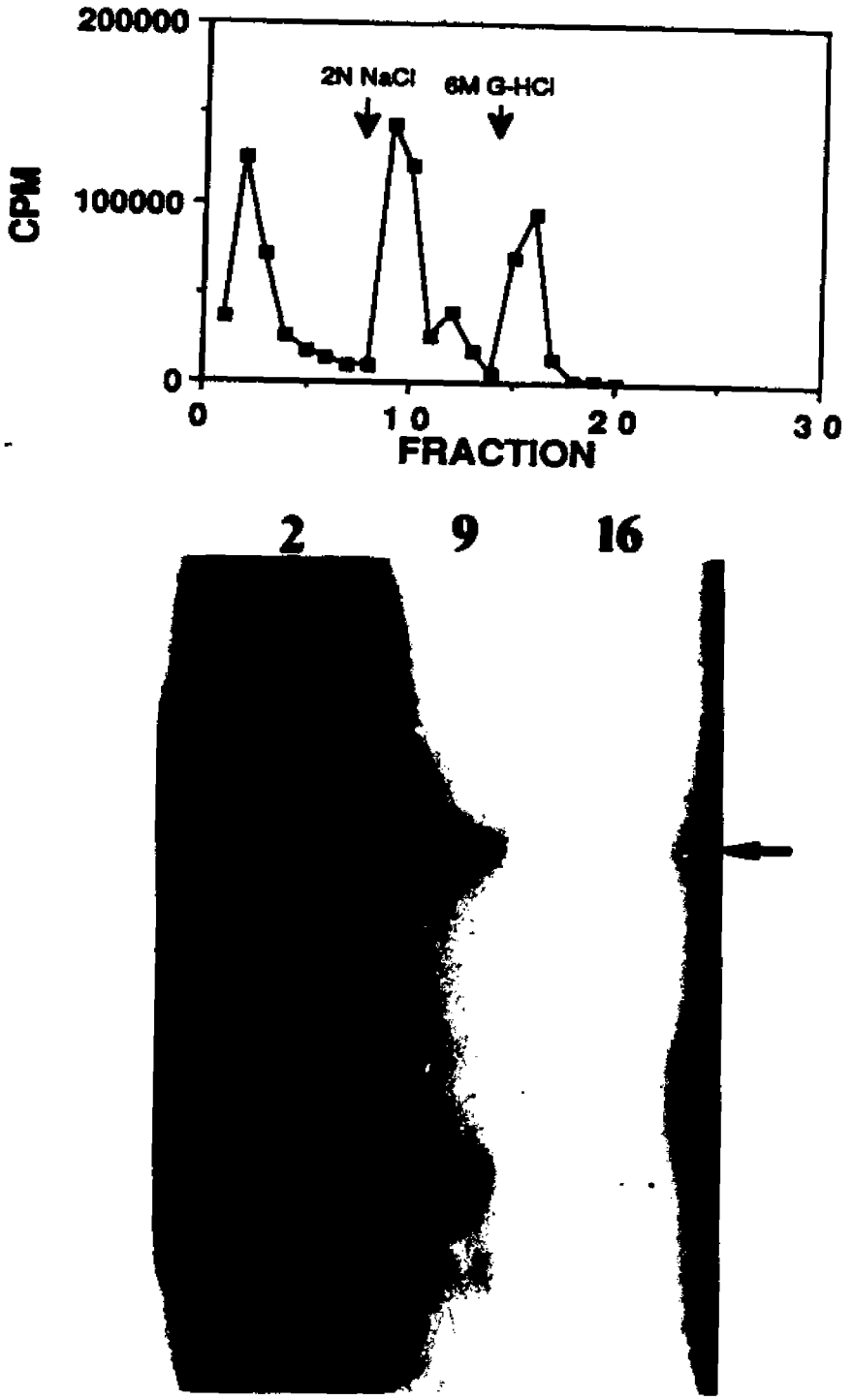


Figure 5.15 **Sepharose-chitooligomer column (I).** Elution with 2N NaCl and 6M guanidine-chloride of radiolabeled chitovibrin concentrate from Sepharose-chitooligomer column. A= radioactivity measured on 100µl of sample. B= autoradiography on fractions 2, 9 and 16. Arrow points at chitovibrin band.

Figure 5.16, and it shows a small peak eluting with 6 M guanidine hydrochloride, which suggests that only a small fraction of "binding-able" chitovibrin elutes in the flowthrough fractions.

To further examine the specificity of binding, fractions 15 and 17 (*Fig.* 5.15) were re-chromatographed on the buffer-washed Sepharose-chitooligomer column, indicating that approximately 40 % of the label bound again with high affinity (*Figure* 5.17, panel A). Fractions eluted with guanidine-hydrochloride were dialyzed and reapplied to the column. The binding under these conditions appears to be highly specific (*Figure* 5.17, panel B), suggesting that the binding capacity of chitovibrin, lost by the 6 M guanidine-hydrochloride elution, may be recovered through dialysis.

5.9 Isoelectric pH of chitovibrin

To achieve a more successful separation of the 2 proteins by ion-exchange chromatography, the optimal conditions must consider the charge of the proteins at the pH of the elution buffer. The isoelectric point of the proteins, a characteristic of a protein, was determined through an isoelectric focusing experiment, as described in Methods. Ten microliter of a 250 µg/ml sample of purified chitovibrin was chromatographed on a flat bed IEF experiment. The results on *Figure* 5.18 indicate that chitovibrin has a pI close to 3.6.

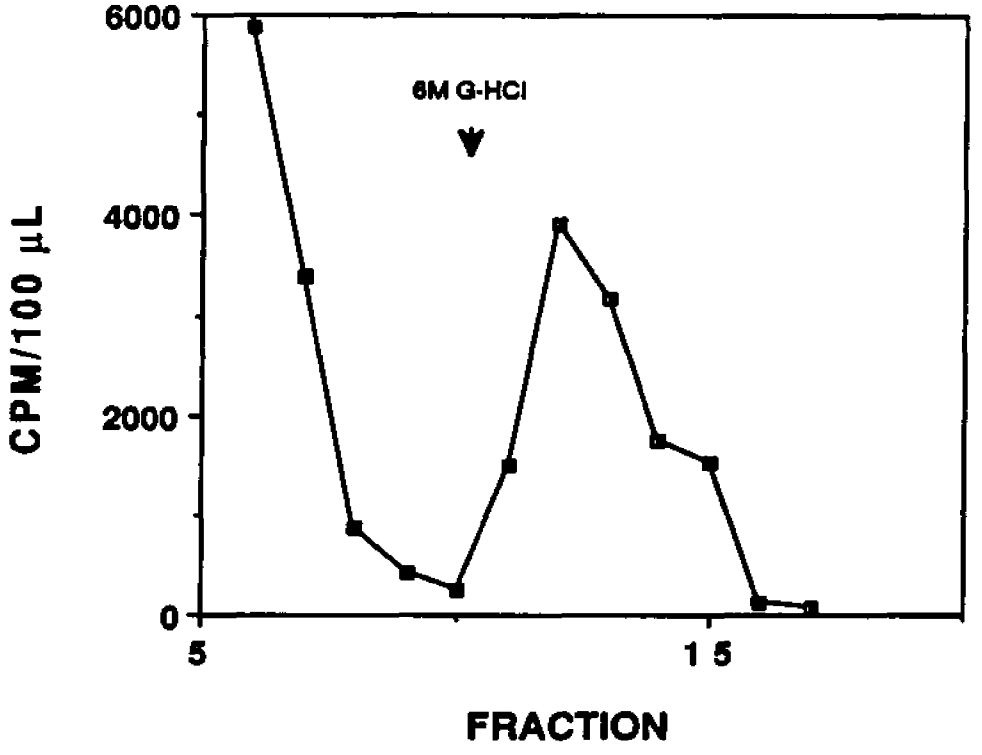


Figure 5.16 **Sepharose-chitooligomer column (II). Fraction 2 (peak fraction in the flowtrough from Figure 5.15) re-chromatographed on Sepharose-chitooligomer column. Sample was eluted with 6M guanidine-hydrochloride.**

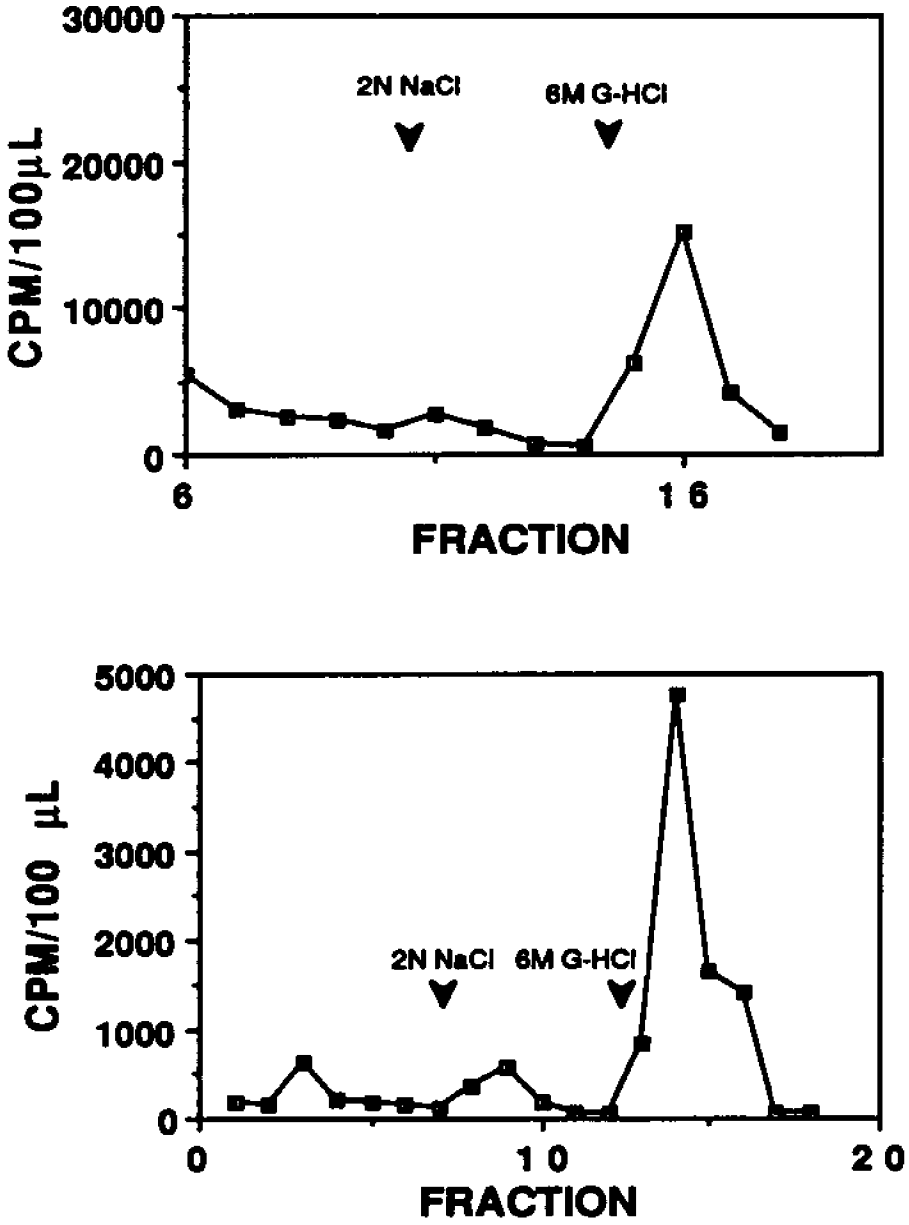


Figure 5.17 **Sepharose chitooligomer column (III). A= Pool of fractions 15 and 17 from experiment in Figure 5.15, re-loaded onto Sepharose-chitooligomer column. B= Fraction 16 from panel A, re-chromatographed on Sepharose-chitooligomer column.**

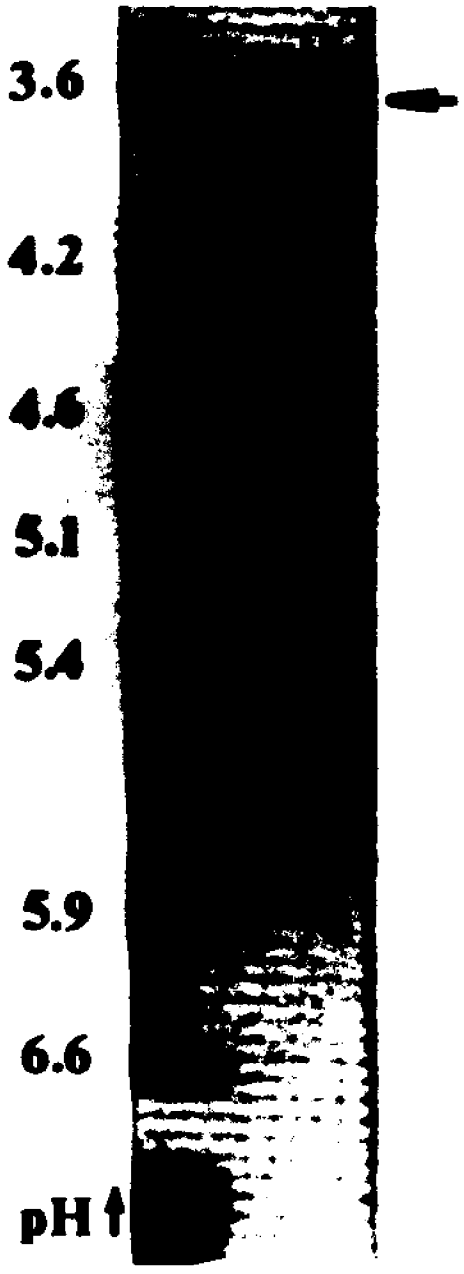


Figure 5.18 **Isoelectric pH of chitovibrin.** The pI value of chitovibrin was examined on a flat-bed isoelectric focusing assay. The arrow points to chitovibrin band.

5.10 Chitovibrin-chitin binding: pH dependency

Binding of chitovibrin to chitin substrate was examined at different pH, ranging from 3.6 to 10.8. The assay was performed with radiolabeled, purified chitovibrin as described in Methods. Results were obtained by scanning autoradiographies of the bound proteins. *Figure* 5.19 shows the pH-dependent binding profile of chitovibrin.

5.11 Binding to neoglycolipids

5.11.1 Binding to neoglycolipids (I)

Binding of radiolabeled chitovibrin to neoglycolipids was examined at pH 8.8 on 96-well plates, as described in Methods. Each well received an aliquot of 10 µl of neoglycolipids. The sugar moiety in the glycolipid ranged from 3mer to 12mer. A sample of fraction 60 (*Figure* 5.4) was radiolabeled in the presence of chitin oligomers, and 50 µl of the labeled protein (approximately 105 cpm) were used for the binding in each well. A neoglycolipid prepared with lactose was used as a negative control. The results on *Figure* 5.20 are expressed in cpm/µM. The binding revealed to be oligomer size-dependent.

5.11.2 Binding to neoglycolipids (II)

Binding of chitovibrin to neoglycolipids made from chito-10mer (NGL-10) and chito-11mer (NGL-11) was examined at pH 7

in the presence of increasing concentrations of chitin oligosaccharides, ranging from 0.1 μM to 30 mM. The results of duplicate experiments are shown in *Figure* 5.21. Results for both substrates appear to indicate that chitovibrin binding is affected by the presence of chitin oligomers.

5.12 Molecular weight of chitovibrin

The molecular weight of chitovibrin was determined through mobility on gel electrophoresis, as compared to molecular weight markers. The apparent molecular mass observed is 134 kDa, as shown in *Figure* 5.22.

5.13 N-terminal sequence and sequence homologies

Chitovibrin was analyzed for its N-terminal sequence, after transfer onto polyvinylidene fluoride membrane, according to the procedure described by Matsudaira (1987). The sequence was obtained at Baylor College of Medicine, Houston TX. The sequence was compared with sequences in the Genebank, and the best fitting sequences analyzed for significance. The best similarity is obtained with the sequence for a sucrase from *Vibrio alginolyticus*, shown in Appendix A.2.

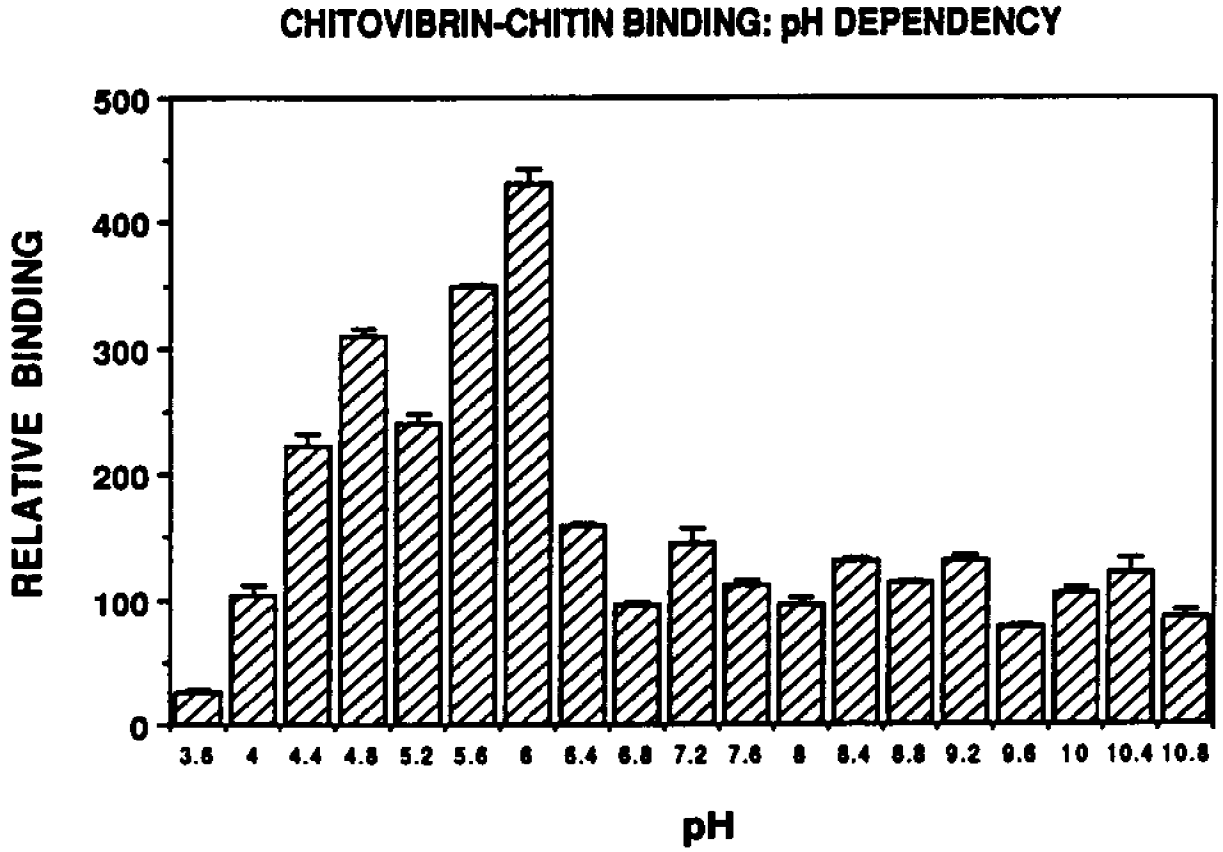


Figure 5.19 **Binding of chitovibrin to chitin: dependency of pH. Binding of chitovibrin to chitin was examined at pH ranging from 3.6 to 10.8. Analysis was done through scanning autoradiographies of the bound proteins.**

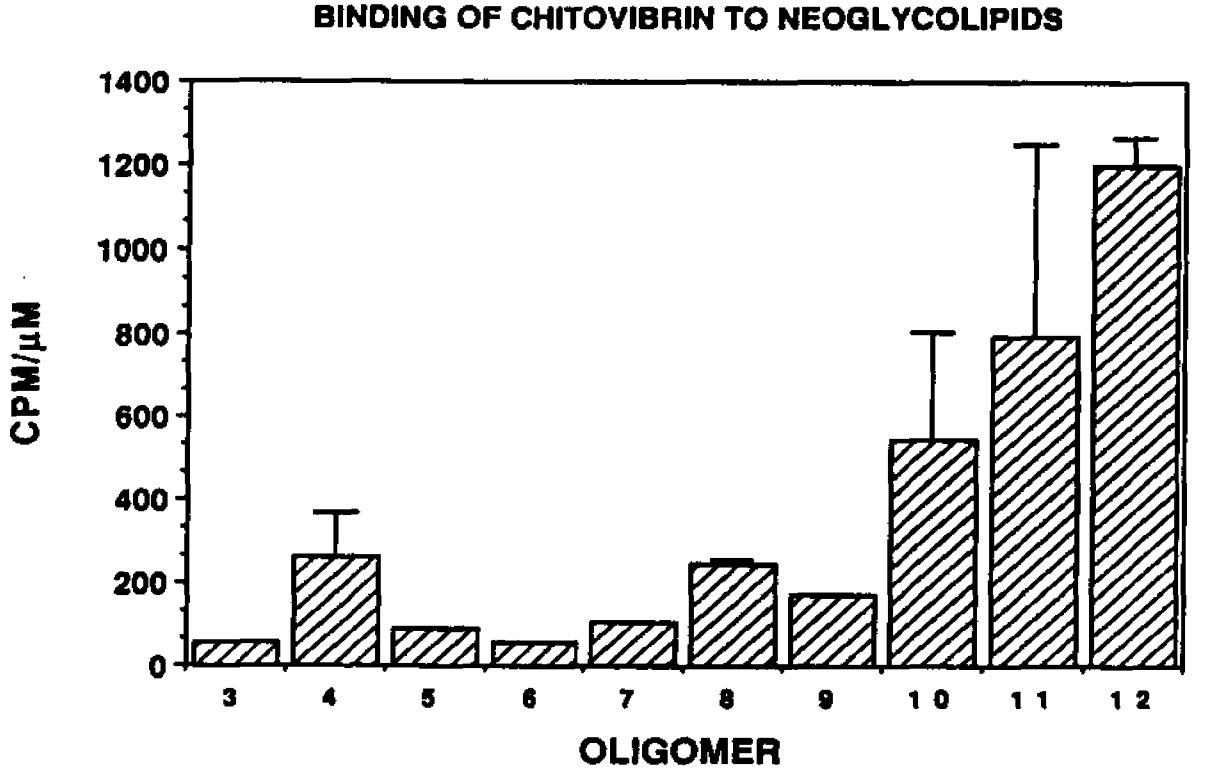


Figure 5.20 **Binding of chitovibrin to neoglycolipids (I). Binding to neoglycolipids was measured on 96-well plates, using lactose-neoglycolipid as control.**

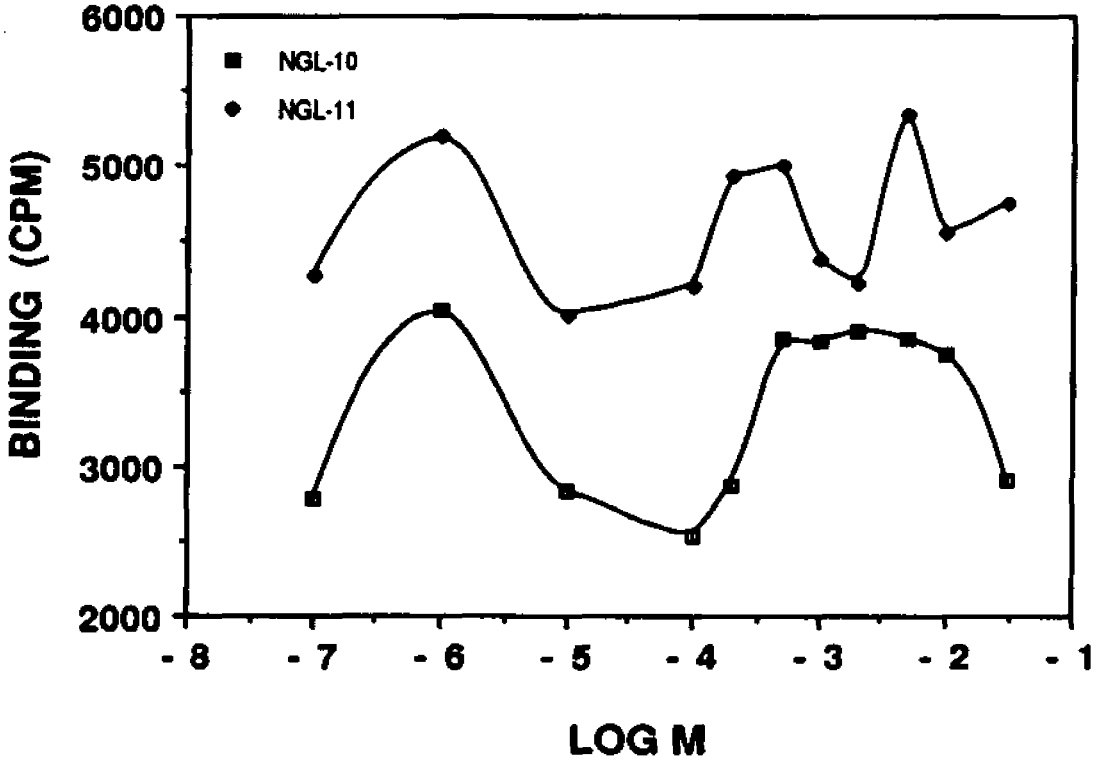


Figure 5.21 **Binding of chitovibrin to neoglycolipids (II).** Binding of chitovibrin to neoglycolipids prepared from chito-10mer and chito-11mer was examined at pH 7 and increasing concentrations of chitooligosaccharides.

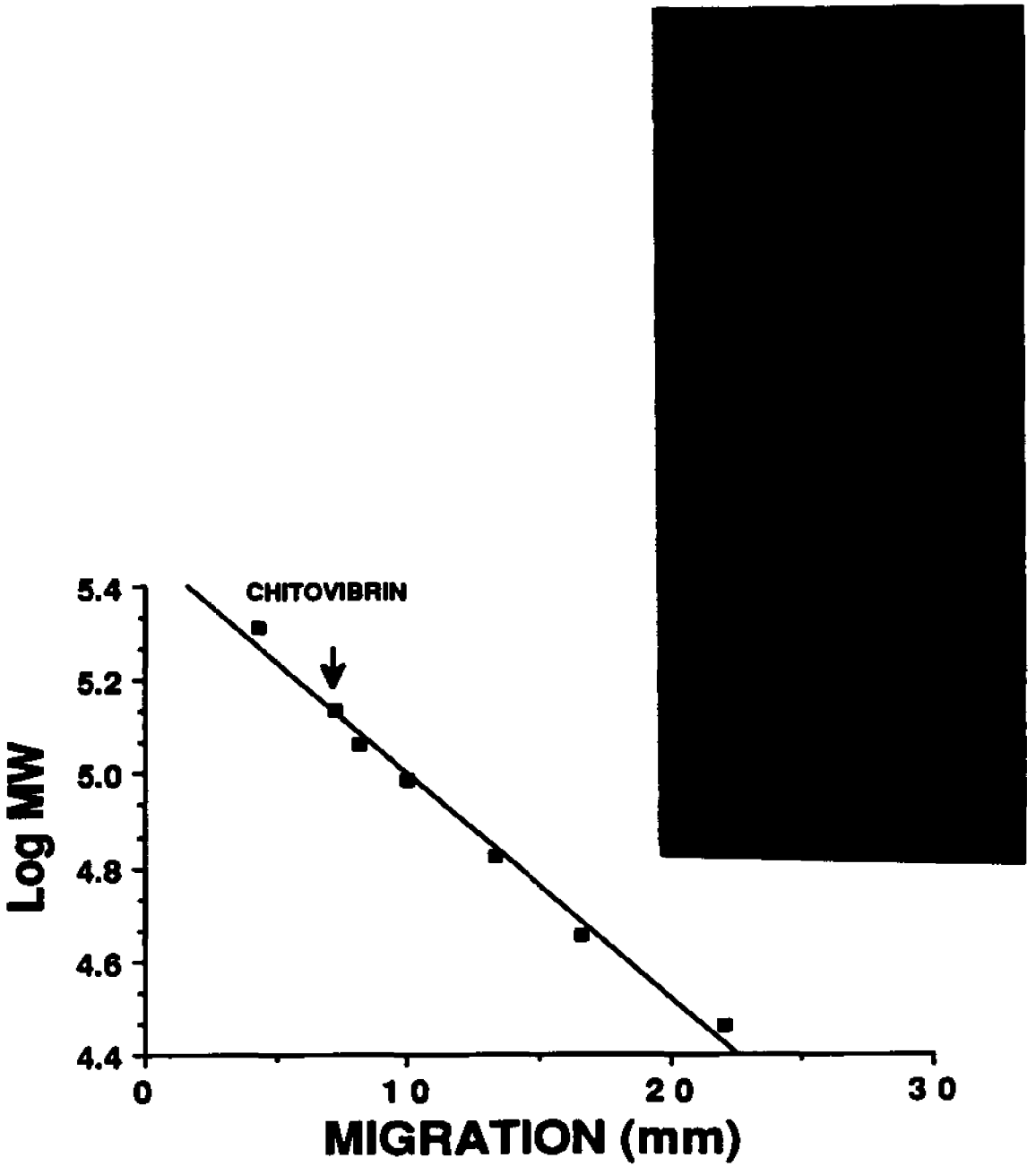


Figure 5.22 **Molecular mass of chitovibrin.** Molecular mass of chitovibrin (134 kDa) was determined through relative mobility on SDS-polyacrylamide gel electrophoresis.

```
SCORES      Initl: 41 Initn: 41 Opt: 53
             40.0% identity in 25 aa overlap

                                                  10        20
Chitov                                    AVDAAPLEVYDSNKVYNGGDQVQHE
                                          :|:   | : |||: ||:||: |:::
Ju0091 FYQWTPVGPVHGMKYWYHLSTKDFIHFTDHGVGLHPDQDYDSHGVYSGGALVENNQVLLF
            70        80        90       100       110       120
```

Figure 5.23 **N-terminal sequence.** The N-terminal sequence of chitovibrin was observed to display some homology with the sucrase sequence from *Vibrio alginolyticus*.

Chapter 6

Conclusions and future prospects

A chitin hydrolase and a chitin-binding protein termed chitovibrin, were secreted (among other polypeptides) by *Vibrio parahaemolyticus*, when the growth medium was supplemented with chitin, oligomers of N-acetyl glucosamine, and to some minor extent, after the addition of other saccharides. The chitinase functions as an endo-chitinase, cleaving glycosidic bonds at random in the middle of chitin molecules and releasing di-N-acetylchitobiose. So far, the role of chitovibrin is unclear, although results which are presented here, together with similarities to other glycosyl hydrolases, may allow speculation on its function in the growth of the *Vibrio* cell.

6.1 Chitinase

A 95 kDa chitinase is expressed in *Vibrio parahaemolyticus* after adding chitin or oligomers of N-acetylglucosamine to the growth medium. Other sugars, which are not a substrate for the enzymes, can also induce in *V. parahaemolyticus* the synthesis of chitin hydrolases, but at a highly reduced level.

Upon hydrolysis of chitin or chitin oligosaccharides, endochitinases generate mainly N-diacetyl chitobiose. Some endochitinases have been reported to express exoenzyme activity

to certain extent (Koga *et al.*, 1983, Funke and Spindler, 1989). The chitinase expressed by *V. parahaemolyticus* seems to show exclusive endo-character, as suggested by results of incubation with the artificial substrate 4-methylumbelliferyl-N,N',N''-triacetyl-ß-chitotrioside (Chapter 4).

6.1.1 Comparative analysis

Molecular Mass:

Endochitinases from different sources seem to vary widely in their physical and chemical characteristics. Some organisms appear to produce more than one chitinase species as determined by purification procedures (Pedraza-Reyes and Lopez-Romero, 1989) or by immunological methods (Koga *et al.*, 1983, Fuchs *et al.*, 1986, Nasser *et al.*, 1988). A possible explanation for the appearance, in plants, of different classes of chitinase was given by Shinshi *et al.* (1990). By studying the structure of the chitinase gene in tobacco, these authors suggested that different chitinase genes can arise by transposition of a sequence encoding a cysteine-rich domain. *Vibrio parahaemolyticus* appears to produce just a 95 kDa chitinase, which is unstable prior to purification, probably because of proteinase activity in the crude extract. By losing a 30 kDa fragment from the carboxyl terminus, the chitinase degrades to a 65 kDa peptide that exhibits the same specific chitinolytic activity as the full-length enzyme. The smaller size form of the enzyme is

barely recognized by polyclonal antibodies raised against the 95 kDa polypeptide. The 65 kDa chitinase elutes before the 95 kDa form from an anion-exchange column upon increasing salt concentration. This result is consistent with the isoelectric pH calculated for either chitinase forms from the complete sequence of the chitinase (Appendix A.2). A more thorough characterization of the 65 kDa form of the enzyme is needed to better understand the effect that the loss of the 30 kDa fragment has on the enzyme.

Bacterial chitinases are generally larger than the enzymes originating from plants. Chitinases isolated from yam (Tsukamoto *et al.*, 1984), potato (Kombrink *et al.*, 1988), wheat germ (Molano, *et al.*, 1979) and bean (Mauch and Staehelin, 1989) have all masses in the range of 30 to 40 kDa. Fungal chitinases isolated from *Mucor rouxii* (Pedraza-Reyes and Lopez-Romero, 1989) showed masses of 24.2 and 30.7 kDa. Insect chitinases have sizes that range from 48,000 to 75,000 (Chen *et al.*, 1982, and Koga *et al.*, 1983).

The *V. parahaemolyticus* chitinase is larger than most of the bacterial endochitinases reported so far. A 52 to 58 kDa enzyme was isolated from *Serratia marcescens* by Roberts and Cabib (1982). A slightly larger chitinase from a *Vibrio* species was reported by Ohtakara *et al.* (1979). Chitinases from different *Streptomyces* species ranged from 19 kDa to 33 kDa (Okazaki and Tagawa, 1991). The only report of a bacterial chitinase with a molecular mass of 110-115 kDa has been detected in *Aeromonas*

hydrophila (Yabuki *et al.*, 1986), a species that also belongs to the family *Vibronaceae*.

Activity-pH:

Chitinases from different sources show a wide range of optimum pH. Chitinases from pupal haemolymph of *Manduca sexta* (Koga *et al.*, 1983), from yam (Tsukamoto *et al.* (1984) and the chitinase purified by Ohtakara *et al.* (1979) from an undetermined *Vibrio* species displayed activity* at pH ranging from 5 to 10. In general, chitinases show one peak of maximum activity which usually lies between pH 5 to pH 7 (Okazaki and Tagawa, 1991, Chen *et al.*, 1982, Yabuki *et al.*, 1986). On the other hand, the optimal pH shown by chitinases from animal sera is between 1.5-2 (Lundblad *et al.*, 1974, 1979). As shown on *Figure* 4.7, *Vibrio parahaemolyticus* chitinase exhibits two pH optima at 6 and 9. This unusual characteristic for a chitinase has only been described before by Tsukamoto *et al.* (1984) for the enzyme isolated from yam.

It is difficult to rationalize the mechanism by which the chitinase can catalyze the hydrolysis of glycosidic bonds from chitooligomers at basic and acidic pH. The glycosidic bond is broken upon protonation of the glycosidic oxygen followed by nucleophilic attack of the anomeric carbon by water. Thus, most hydrolases function at neutral or acidic pH. Under these conditions

* ≥40% of maximum

the proton donor is generally a Glu, Asp or His residue. At pH 9, the proton donor may be a tyrosine or a lysine.

Wheat-germ agglutinin (WGA) and lysozyme are two of the best characterized proteins having similar sugar-binding specificity as chitinase. WGA binds to di-N-acetylchitobiose and higher chitin oligomers without effecting hydrolysis. The structure and binding characteristics of WGA have been worked out by Wright (1984) through X-ray crystallography. Lysozyme is a glycosidase (EC 3.2.1.17) that has been studied extensively. It hydrolyzes peptidoglycan which is a polysaccharide from bacterial cell walls formed by alternating N-acetylmuramic acid and N-acetylglucosamine sugars, linked by ß(1->4) glycosidic linkages. This polysaccharide is structurally very similar to chitin, the only difference being an additional lactyl side chain attached to C-3 hydroxyl on every second GlcNAc residue. Some lysozymes reportedly display chitinase activity. Hydrolysis of an artificial chitin-homolog by lysozyme has been determined on hen egg-white (HEW) lysozyme by Yang and Hamaguchi (1980). A chitinase from cucumber has been reported to exhibit lysozyme activity. (Majeau *et al.*, 1990). Hara *et al.* (1989) showed that the binding to the substrate by chitinase from *Streptomyces erythraeus* is similar to that of HEW and *S. erythraeus* lysozymes. The reported results seem to indicate that the lactyl side chain is not a determining factor in the substrate binding and specificity for these proteins.

To try and understand the mechanism of substrate binding and hydrolysis for the *Vibrio* chitinase, similarity analysis of sequences in the Swissprot data base, using the FASTA and BESTFIT programs, was performed. No significant sequence homologies between lysozyme and either WGA, *Vibrio* chitinase, or other chitinases was observed. WGA displays no sequence homology with *Vibrio* or any other bacterial chitinases, but shows significant similarity to plant chitinases. The chitinase from *Vibrio parahaemolyticus* exhibits high homology with chitinases from *Serratia* and *Bacillus* (Appendix B).

The enzyme from *Vibrio parahaemolyticus* hydrolyses chitin and chitooligomers as an endo-chitinase, releasing N,N'-diacetylchitobiose. The rate of hydrolysis of chitotrimer is very slow, but tetramer and higher oligomers are hydrolyzed at high rates. Drawing a parallel with lysozyme, the conformation of *Vibrio* chitinase may be strained upon binding to substrates consisting of 3 or more residues, which would bring the active site closer to the substrate glycosidic linkage to be hydrolyzed. As it is equally efficient at either pH optima, the chitinase from *Vibrio parahaemolyticus* may contain more than one proton-donor amino acids, active at acidic or basic pH. We might speculate that these amino acids would differentially become part of the active site depending of the pH of the environment which would produce a concomitant alteration in the conformation of the protein.

Isoelectric pH

The chitinase from a stable fly described by Chen *et al.* (1982) and the shrimp enzyme isolated by Funke and Spindler (1989) have pI values of 4.85 and 3.9, respectively. Plant chitinases may exhibit pI values of 3.8 (Tsukamoto *et al.*, 1984) to 8.7 (Verburg and Huynh, 1991). Isoelectric pH values ranging from 3.1 for *Streptomyces erythraeus* (Hara *et al.*, 1989) to 8 and higher for different *Streptomyces* species (Okazaki and Tagawa, 1991) have been observed in bacterial chitinases. Chitinase from *Nocardia orientalis* exhibited an unusually high pI of 8.8 (Usui *et al.*, 1987). The pI for the *V. parahemolyticus* chitinase has been calculated from the amino acid sequence to be 4.27.

Substrate specificity

The results from the substrate specificity experiment (*Figure* 4.7) indicate that the chitinase is equally active on chitin oligosaccharides with degree of polymerization of 4 or higher. This result may indicate that the size of the binding site for the enzyme spans a region comparable to the length of a chito-tetramer. The appearence of esentially only di-N-acetyl chitobiose as the product upon incubation of different chitooligosaccharides with the chitinase defines this activity as a chitinase (*Figure* 4.7).

Proof of the endo-chitinase character of the enzyme was provided by the fluorescence results after chitinase incubation with 4-methylumbelliferyl-N,N',N''-triacetyl-ß-chitotrioside. The

enzyme activity, using the fluorescent substrate, was determined following the procedure described by Robbins *et al.* (1988).

Transglycosylation activity was reported by Usui *et al.* (1987) for a chitinase from *Nocardia orientalis.* The pH optimum for the chitinase activity from *Nocardia* was 5.5, but transglycosylase activity was higher at pH 4 and 7. Chito-hexamer was not a substrate for this activity.

Temperature optimum

The optimum activity for crustacean chitinases has been determined to lie between 50°C and 55°C (Funke and Spindler, 1989, and Lynn, 1990). The chitinase isolated from yam by Tsukamoto *et al.* (1984) is unusually heat-stable: the enzyme remains equally active upon incubation at temperatures below 60°C . Chitinase activities purified from bacterial cultures seem to exhibit comparable temperature dependency. As our own results show on *Figure* 4.6, the chitinase from *Streptomyces cineoruber* (Okazaki and Tagawa, 1991) and the chitinase from a *Vibrio* species reported by Ohtakara *et al.* (1979) exhibit maximal activity at 50°C. The heat-stability of chitinolytic enzymes has relevance on industrial chitin-processing plants that consider biodegradative operations.

Structure and Function

Polyclonal antibodies raised against the 95 kDa chitinase bind very poorly the 65 kDa form, thus suggesting that either the loss of the 30 kDa peptide drastically affects the comformation of the protein, and/or that the fragment contains most of the antigenic epitopes recognized by the antiserum. Screening of the *Vibrio* genome suggested the existense of only one gene coding for chitinase activity (Dr. Chin-Yih Ou, personal communication).

The 65 kDa enzymatically active peptide seems not to be autoproteolytically generated, since the 95 kDa chitinase appears to become stable upon purification. The loss of the 30 kDa fragment does not appear to diminish enzymatic activity. The existence in the protein of a region comprising one third of its length and which seems to have no relevance to its normal function is puzzling. Homology of fungal cellulases with HEW lysozyme and other ß-1,4-glycoside hydrolases has been established (Yaguchi *et al.*, 1983; Paice *et al.*, 1984), but the discovery of significant sequence homology between the C-terminal 30 kDa fragment and the C-terminus of endoglucanase (EC 3.2.1.4) from a *Bacillus* species (strain N-4) was unexpected, especially since the homologous region does not seem to contain any known function in the endoglucanase from *Bacillus*. Both proteins contain two consecutive tryptophan residues in the homology region. In lysozyme, Trp 62 and Trp 63 are proposed to be hydrogen-bonded to C6 and C3 oxygens of the third residue in

the substrate $(GlcNAc)_3$, respectively. *Bacillus* endoglucanase contains a 24 amino acid stretch with two tandem repeats of 8 amino acids each. Fifty percent of the residues in this section are proline residues.

Since the 95 kDa and 65 kDa chitinases exhibit equivalent activity, the 30 kDa C-terminal fragment might be involved in (i) chitinase secretion or (ii) structural stabilization of the enzyme. Studies on *E. coli* haemolysin, which does not have an N-terminal signal sequence, suggest that the C-terminal domain is required for the translocation of this protein across the plasma membrane (Pugsley and Schwartz, 1985; Gray *et al.*, 1989). *Vibrio* chitinase is synthesized with a 22 amino acid N-terminal signal peptide which appears to contain the necessary information to process its export (Pugsley and Schwartz, 1985). This sequence is removed from the protein during translocation. Moreover, a deletion mutant clone pKS139-2 (variant 3427), being only the N-terminal section of the *V. parahaemolyticus* gene, formed a clear zone in chitin agar, suggesting secretion from *E. coli* was not affected by loss of the 30 K fragment (Dr. Jing-Yi Lo, personal communication). Nevertheless, it has been reported that, apart of the 35 kDa catalytic domain of an endocellulase from *B. subtilis*, the enzyme carries a 16 kDa carboxy-terminal extension. A similar region has been found in endoglucanase EG1 from *B. circulans*. Proteolytic processing of non-essential regions in cellulolytic proteins was observed for native and cloned hydrolases (Beguin, 1990)). The removal of the non-

catalytic region in the native host suggests this domain is not involved in hydrolysis of cellulose. Substitutions in the C-terminal region of EG1 from *B. circulans* reportedly reduced the export of the enzyme drastically, suggesting a role in secretion for the non-catalytic domain (Hansen, 1989). However, when a C-terminal truncated fragment of a *B. subtilis* endoglucanase was cloned into *E. coli*, the organism was able to secrete most of the enzyme, indicating that the region was not required for extracellular secretion in *E. coli*. This information suggests that the 30K, C-terminal fragment of *V. parahaemolyticus* chitinase may be involved in secretion in the native host.

Other Features

The chitinase activity is unusually resistant to high ionic strength, which may be appropriate for a halophilic organism which, at times, may need to survive in brackish waters with high salt concentrations.

The binding of hexose rings is generally mediated by hydrophobic interactions with tyrosine or tryptophan residues, which may account for the high degree of hydrophobicity exhibited by chitinase, as shown in *Figure* 5.9.

Potential use of the robust characteristics of the *V. parahaemolyticus* chitinase in chitin-derived food and

pharmaceutical products, as well as in animal and aquaculture feed, should be considered.

Future prospects

Future research on the *Vibrio parahaemoliticus* chitinase might consider experiments to determine a) the minimal peptide sequence that exhibits chitinase activity b) the structure of the binding/active site c) the chitin-utilization operon, which would also include d) the structure of chitinase inducers.

6.2 Chitovibrin

A protein, produced by *Vibrio parahaemolyticus*, appears to be secreted to the growth medium simultaneously with chitin hydrolases, after being induced in the organism by di-N-acetylglucosamine, chitooligomers or cellobiose. The protein, provisionally named *chitovibrin*, exhibits a strong and specific affinity to chitin and chitin oligosaccharides over a broad range of pH.

6.2.1 Characterization

Chitovibrin, with a molecular weight of approximately 135 kDa, could be purified from the 95K form of the chitinase secreted by the bacterium through selective elution from chitin matrix columns or by gel permeation chromatography. A highly improved separation of chitovibrin could be obtained with the latter method

only if the samples and elution buffers contained a detergent (ß-octylglucoside) to minimize the interaction between chitovibrin and the chitinases. This interaction, probably of hydrophobic nature, may be an important factor in the attachment of *Vibrio* to chitin hydrolases and chitin-containing substrates. Chromatography of a crude preparation on Phenyl-Sepharose CL-4B gel (*Fig.* 5.9) revealed that chitovibrin, as well as the two chitinase forms, have strong hydrophobic character. Chitovibrin exhibits a pI of 3.6 and strong binding activity to chitin and chitin oligosaccharides, which has a maximum at pH 6.

Neoglycolipids have been successfully used to detect and analyze carbohydrate-binding activities (Tang *et al.*, 1985; Stoll *et al.*, 1988). Construction of neoglycolipids of chitin oligosaccharides with D.P.=4 was reported by Lawson *et al.* (1990), but no reports on neoglycolipids derived from chitoligosaccarides larger than tetramer have been published. Binding of chitovibrin to neoglycolipids appears to depend on the size of the substrate and on the concentration of chitin oligomers in the medium. Chitovibrin does not show antigenic crossreactivity with antichitinase antibodies.

Radiolabeling chitovibrin with ^{125}I appears to reduce the binding capacity of chitovibrin. Lucas *et al.* (1985) observed that the amino-terminal region of the ethylene-induced chitinase from bean leaves, which recognized fungal chitin, bears considerable

resemblance to the WGA domain that binds aminoacetylated aminosugars. Amino acid number 30 is a tyrosine and is conserved in WGA, the bean-leaf chitinase and hevein, a protein from rubber tree latex. Tyrosine may be involved in the binding region (and/or active site domain for the chitinase) after analyzing the results from radioiodination of chitovibrin and chitinase (Appendix A.3).

6.2.2 Putative Function

The bacterial glycocalyx is a mass of tangled fibers of polysaccharides that coveres the cell and enables it to adhere to desirable surfaces, ranging from a submerged rock to the intestine of a cow (Costerton *et al.*, 1978). This fibrous envelope has also been implicated in protection against antibacterial agents (Pel *et al.*, 1990). The polysaccharide fibers in the glycocalyx are for the most part negatively charged (Costerton *et al.*, 1978 and 1981). The mode of binding relies heavily on divalent cations and lectins which, in the case of binding to higher-cell polysaccharides, can form a bridge-like structure. The binding activity of chitovibrin appears to be modulated by the concentration of chitin oligosaccharides in the environment (*Figure* 5.21). If this observation is real, it would suggest the presence another sugar-binding site(s). If this were the case, one possible function of chitovibrin might be to form a network through its binding both glycocalyx and chitinases, confining the hydrolytic enzymes and the nutrients released in close proximity to the cell.

Results from induction (*Figures* 5.11 and 5.12) and protein localization experiments (*Figure* 5.2) indicate that several proteins are secreted by *Vibrio parahaemolyticus* upon chitin addition to the growth medium. The results in *Figure* 5.8 suggest that the affinity-purified antichitinase antibodies bind several of the proteins in the crude preparation. The Western-blot results shown in *Figure* 5.6 indicate that chitovibrin is not recognized by the antichitinase antiserum. Other proteins in the sample showed a positive reaction. Especially strong was the recognition of two polypeptides with approximate masses of 37 kDa and 53 kDa, which could be fragments of the 95K form of chitinase. No precipitin line was observed when the antigenicity of chitovibrin was tested against the antichitinase antibodies on Ouchterlony double-diffusion (*Figure* 5.7). It can be speculated that the proteins induced by chitin may have either chitin-hydrolysis or chitin-binding activity. Furthermore the proteins may aggregate around the growing cells to keep them close to the nutrients after substrate hydrolysis. It has been reported that for some bacterial cellulases, the various components of the activity are found in tightly associated multimolecular complexes (Wu *et al.*, 1988; Beguin, 1990). The cellulase complex of *Clostridium thermocellum* has revealed at least 14-18 different polypeptides forming a very stable extracellular structure termed cellulosome (Lamed *et al.*, 1983). At least 8 of the cellulosome components exhibit cellulase activity. A non-catalytic binding factor seems to mediate the high affinity of the cellulosome for cellulose. This factor, termed S1 by

Lamed *et al.*, showed a molecular mass of 210 kDa. This binding factor could be detected on the cell surface and in the extracellular medium, did not exhibit cellulolytic activity. Immunoprecipitation experiments, using antibodies raised against Sl, coprecipitated cellulolytic activity. A 250 kDa non-catalytic binding factor (SL) was described by Wu *et al.* (1988) in a different strain of *C. thermocellum*. The factor did not exhibit detectable activity, and whether it is a cellulolytic enzyme or an anchoring factor remains to be determined.

The association of cellulase activity and protein is not limited to *Clostridium thermocellum*. Aggregates with masses ranging from 1.5 x 10^6 to >4 x 10^6 Da have been reported in rumen bacteria, according to a review by Ljungdahl and Eriksson (1985). In the case of *C. thermocellum*, the size of the cellulosome was reported to have a size of 6.5 x 10^6 Da (Wu *et al.*, 1988).

Since there seem to be some structural resemblances between a few different glycosyl hydrolases (above), it is possible to speculate that chitin-induced polypeptides in *Vibrio parahaemolyticus* associate to form a complex with similar morphology than the cellulosome in *Clostridium*. The apparent size of the putative aggregate in *V. parahaemolyticus* might be smaller than the cellulosome as (i) there is probably only one chitinase-encoding gene in *V. parahaemolyticus*, (ii) results from gel-permeation experiments (*Figures* 5.3 and 5.5) seem to indicate the

association may be weaker in the *Vibrio* proteins, and (iii) the polypeptides in *V. parahaemolyticus* seem to have an overall smaller size. Further research is needed to determine the validity of this suggestion.

In *Vibrio harveyi*, Yu *et al.* (1987) proposed the expression of an unusual lectin with broad sugar specificity and which would be part of a cellular adhesion-deadhesion apparatus. The system would allow the constant monitoring of the surrounding environment, comprising a nutrient sensorium. By its revealed characteristics, chitovibrin might form part of a similar apparatus in *V. parahaemolyticus*, specifically directed to the detection and assimilation of nutrients originating from chitin.

Future prospects

Future work may contemplate the cloning and sequencing of chitovibrin as well as the determination of its binding characteristics and binding site (by site-directed mutagenesis). Localization experiments might be accomplished by EM studies through the use of immunogold-labeled antichitovibrin antibodies.

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Appendix A

Appendix A.1

Growth media for cultures of *Vibrio parahaemolyticus*

Inocula were generally grown on LB or 804 medium. Culture media* were prepared supplementing 804 medium with 0.5 % swollen chitin. 804 or 9MCA medium, supplemented with varying concentrations of saccharides were used for induction studies.

Per liter:

	LB	804
Tryptone	10 g	1 g
Yeast extract	5 g	1 g
KCl	--	0.75 g
NaCl	10 g	23.4 g
$MgSO_4 \cdot 7H_2O$	--	6.9 g

	M9CA	
Na_2HPO_4	6 g	
KH_2PO_4	3 g	
NaCl	0.5 g	
NH_4Cl	1 g	
Tryptone	2 g	Adjust pH to 7.4, autoclave, and then add:
1 M $MgSO_4$	2 ml	
20% glucose	10 ml	
1 M $CaCl_2$	0.1 ml	Sterilized separately by filtration or autoclaving

* From Maniatis *et al.*, 1982.

Appendix A.2

Amino acid sequences of chitinase and chitovibrin

Chitinase

The complete amino acid sequence of chitinase was deduced from DNA sequence obtained by Dr. Jing-Yi Lo, while working in our laboratory.

```
  1  mirfnlcaag valalssgaa vaaptapsvd mygsnnlqfs kielametts
 51  gyydmvkyhd qakitvkfnq wsgtpgdtyn iyfdgvkvat gaitggqtta
101  tfdygqggly qmeieacdat gcsksapaei tiadtdgshl kpltmnvdpn
151  nktyntdpsv vmgtyfvewg iygrnytvdn mpadnlthil ygfipicgpn
201  esvksvggns fnalqtacrg vpdyevvihd pwaaynksfp qagheystpi
251  kgnyamlmal kqrnpdlkii psiggwtlsd pfydfvnkan rdtfvasvkk
301  flktwkfydg vdidwefpgg ggaaadkgdp vndgpayval mrelramlde
351  leaetgrtye ltsaigvgyd kiedvnyada vqymdyifam tydfyggwnn
401  vpghqtalyc gsfmrpgqcd gsgvdengea ykgpaytadn giqlllaggv
451  panklvlgta mygrgwegvt pdtltdpndp mtgtatgklk gstaqvweeg
501  vidykgiksf mlganntgin gfeygydaqa eapwvwnrtt gelitfddhr
551  svlakgsyak slglaglfsw einadngdil namhegmagg vvpqpnrkpt
601  aaagadqsvt gpasvvldgs lskdsdgtia syvweqvsgt avvlaganta
651  kasfdaaevt veeqltfklt vtdnegatas dlvvvtvkpa gvvdpvnnap
701  vaqvvapata nagdvvvvda sassdadndt ltfnwtlpqg lnatvngskv
751  tftaaeypqd tslsftvsvs dgkasssasa tvvvakhttd pnpgtcdnaw
801  dasavytggn qvthagktwe akwwtqeddp sksgewgvwk evgvancn
```

Figure A-1 **Deduced amino acid sequence of chitinase from *Vibrio parahaemolyticus*.**

Homology between chitinase sequences

Sequence homology between chitinases from *Vibrio parahaemolyticus* (A), *Serratia marcescens* (B) and *Bacillus circulans* (C). Sequence comparisons were obtained through computer programs BESTFIT and FASTA on sequences in the SwissProt data base.

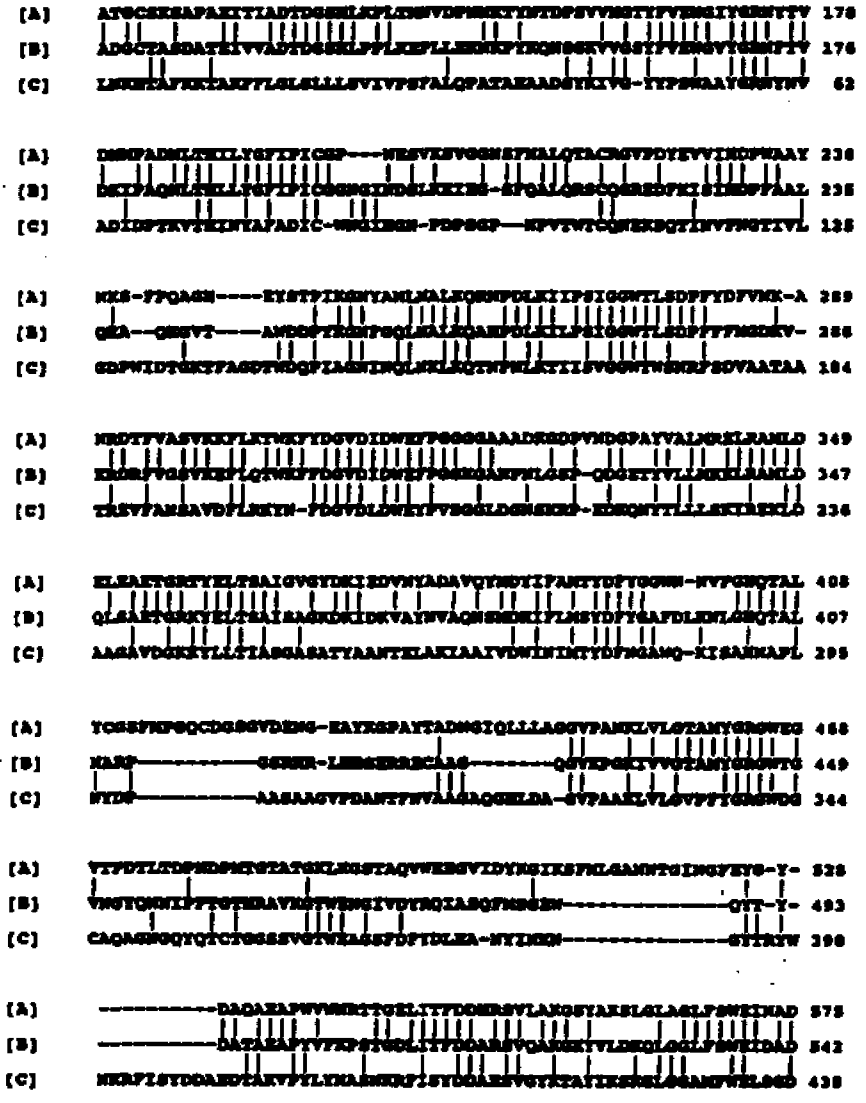


Figure A-2 Sequence homology between *Vibrio, Serratia* and *Bacillus* chitinases.

N-terminal sequence of chitovibrin

After SDS-polyacrylamide gel electrophoresis, a crude protein extract was transferred to polyvinyledene fluoride (PVDF) membrane, and the chitovibrin band subjected to N-terminal analysis. The 25 amino acid sequence obtained, with uncertainty for the last residue, was

1 **A V D A A P L E V Y D S N K V Y N G G D Q V Q H (E)** 25

Appendix A.3

Radiolabeling of chitovibrin

When labeling chitovibrin with the radioisotope ^{125}I, binding of the protein to chitin seemed to be impaired, as is suggested from the disparity of binding observed in the results presented in *Figures* 4.2 and 5.13, compared with results in *Figure* 5.15. Chitovibrin appears to lose its chitin-binding capacity to a great extent. Under the conditions of the labeling, only tyrosine residues are covalently bound to iodine. If one or more tyrosine residues are located near, or being part of the chitin-binding site of the protein, the presence of bulky iodine isotope molecules could prevent chitin or chitin oligomers from binding to the protein.

Considering that (i) the binding site of wheat germ agglutinin, which binds to chitin and chitin oligosaccharides, exhibits tyrosyl residues in the binding site (Wright, 1984 and 1987), (ii) the proteins associated with chitin in crawfish shells reveal an unusually high (69% in mass) content of tyrosine (No *et al.*, 1989), and (iii) many tyrosyl residues seem to be conserved in chitinases that display strong homology with chitinase from *Vibrio*, thus suggesting structural or functional relevance of these residues (Appendix A.2), then the diminished binding capability could be produced by the interference in the binding site through attachment of the radioisotope.

To test this possibility, a sample of chitovibrin was radiolabeled with iodine ^{125}I in the presence and absence of chitin oligomers. Aliquots 100 µl aliquots of a pH 7 chitovibrin solution (15 µg or 0.1 nmoles) were incubated with 1 mCi ^{125}I with (+) or without (-) the addition of 5 µl of an 80 µg/µl solution of chitin oligomers (approx. 0.4 µmoles). The labeling proceded as described in Methods. Chromatography of the labeled proteins on a 9.1 ml, disposable G-25 gel column (10 x 1.5 cm) is shown in *Figure* A-4. Proteins analyzed by electrophoresis on SDS-polyacrylamide gel, seemed to indicate that more protein was labeled in the absence of chitin oligomers. Analysis of the bound label by a filter assay on labeled proteins reveals that at least half of the radioactivity of the chitovibrin labeled in the presence of chitin oligomers was non-covalently bound, as shown in *Figure* A-5.

Samples of labeled chitovibrin (in the presence or absense of chitin oligomers) were dialyzed and examined on SDS-polyacrylamide gel electrophoresis (*Figure* A-6). Results appear to indicate that labeling with ^{125}I interferes with the chitin-binding capacity of chitovibrin. Labeling of cloned chitinase with ^{125}I also resulted in lower activity for proteins labeled in the presence of chitin oligosaccharides (not shown).

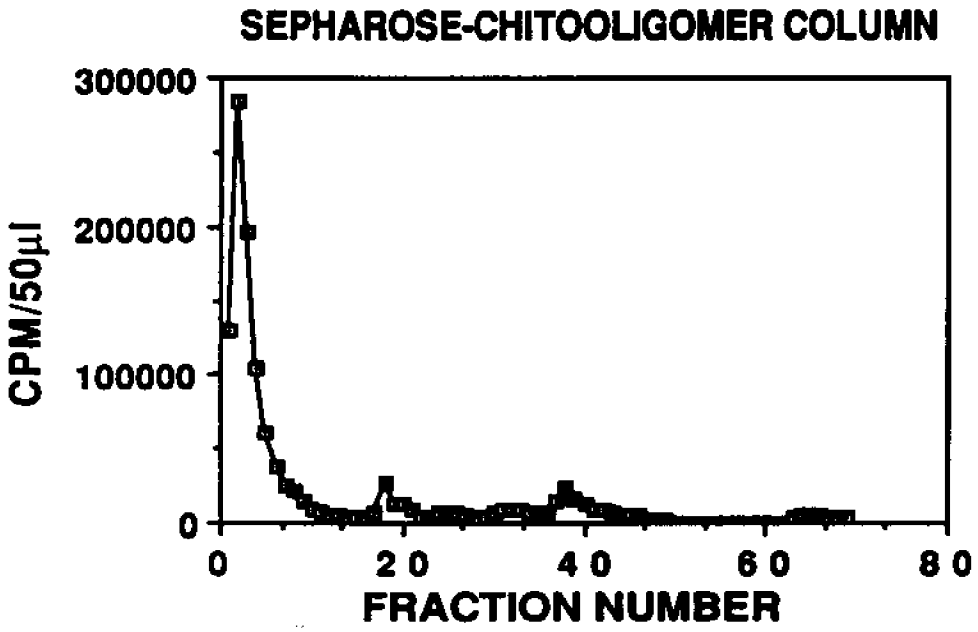


Figure A-3 Labeled chitovibrin on Sepharose-Chitooligomer column. (see *Figure* 5.15)

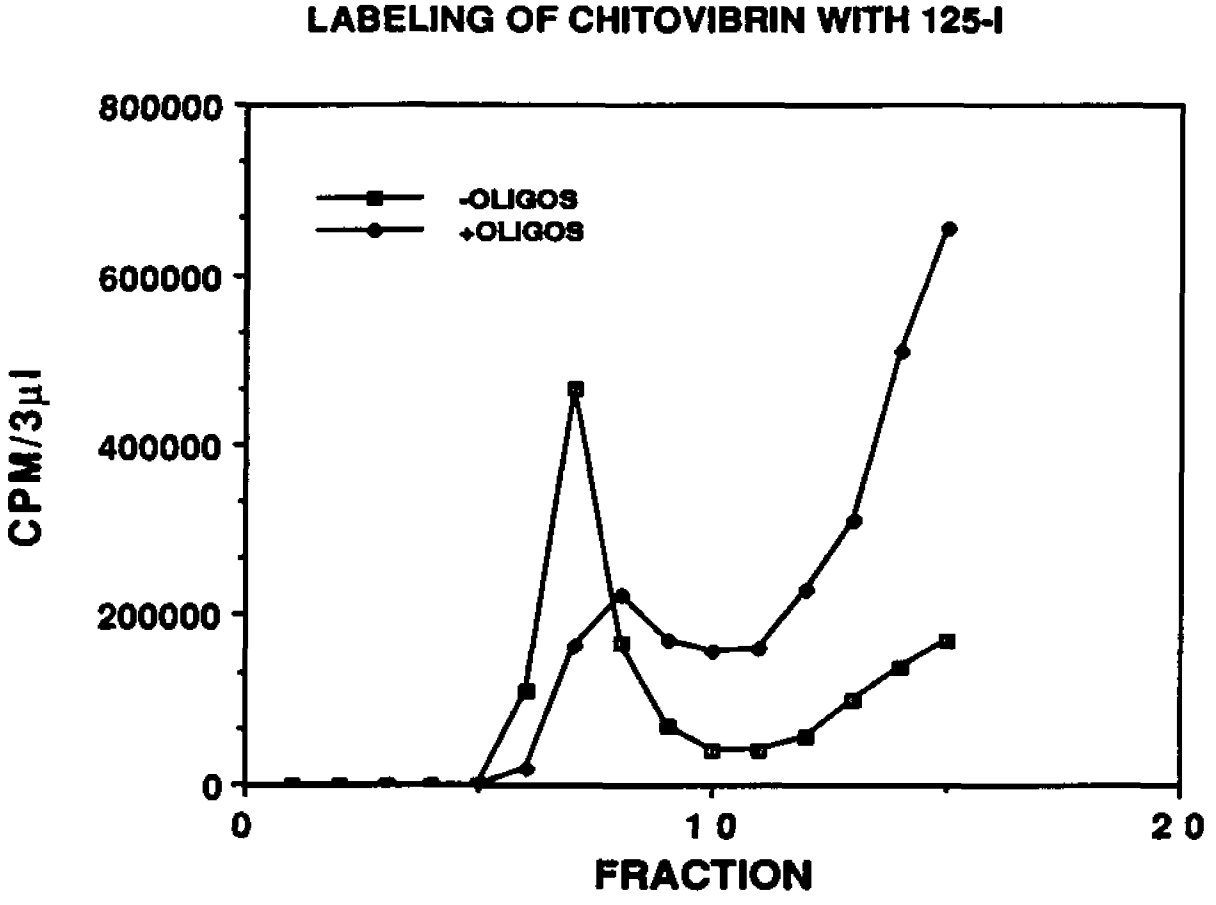


Figure A-4 **Radiolabeling of chitovibrin (I).** Aliquots of chitinase preparation were radiolabeled in the presence (+) and absense (-) of chitin oligosaccharides.

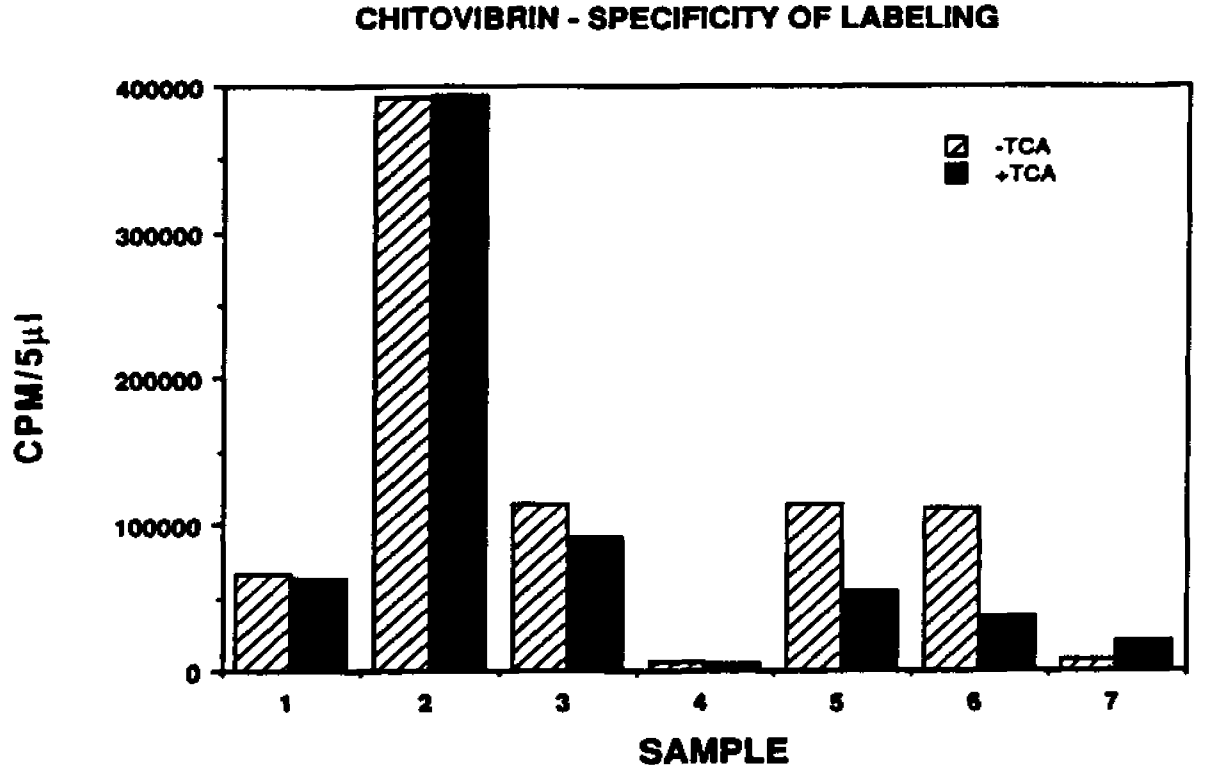


Figure A-5 **Specificity of labeling.** TCA-filter assay of chitovibrin, labeled in presence (+) or absense (-) of chitin oligosaccharides (see text). Samples 1, 2 and 3 correspond to fractions 6, 7 and 8 (- oligos) in *Figure* A-4. Samples 4, 5, 6 and 7 correspond to fractions 6, 7, 8 and 9 (+ oligos) in Figure A-4.

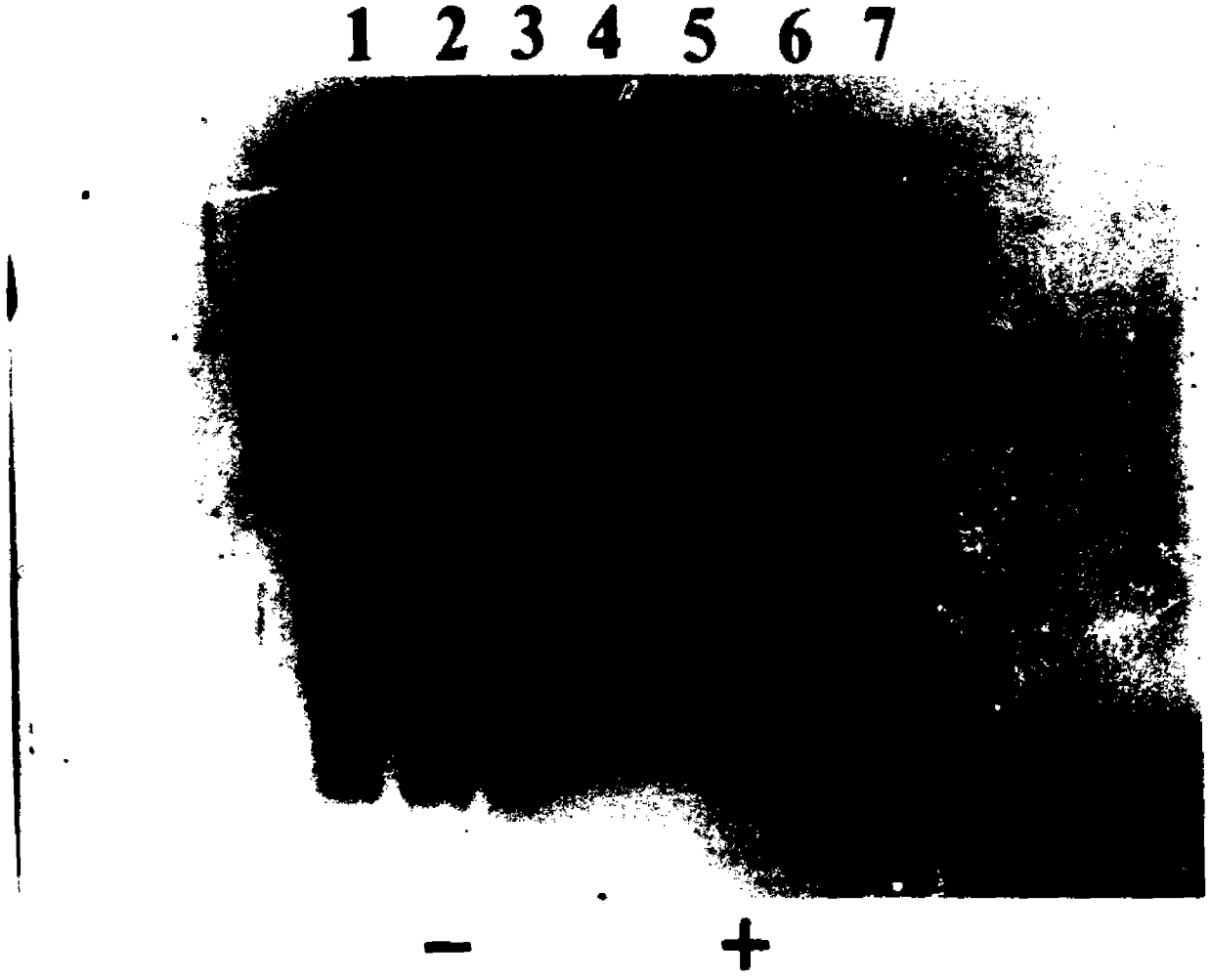


Figure A-6 **Radiolabeling of chitovibrin (II).** Samples described in *Figure A-5* chromatographed on 6 % SDS-polyacrylamide gel electrophoresis, after dialysis. Samples contained equal cpm.

Appendix A.4

Analysis of acetylation of chitin

The degree of acetylation of chitin was examined on chitin subjected to different treatments Three methods to measure aceylation are based on IR spectroscopy, according to a) Domszy and Roberts (1985) and Moore and Roberts (1980), b) Shimahara and Takiguchi (1988), and c) Sannan *et al.*, (1978). The last method is based on the formation of N-salicylidene chitosan (Neugebauer, 1989; Domszy and Roberts, 1985). Table A-1 shows degree of acetylation of chitin samples, as measured by these four methods.

Table A-1

% ACETYLATION

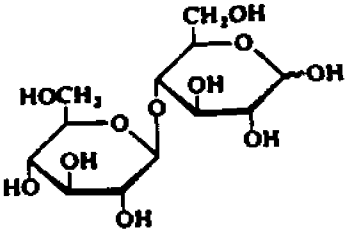
METHOD	REGENERATED CHITIN	REGENERATED CHITIN + RE-N-ACETYLATION 1 HOUR	REGENERATED CHITIN + RE-N-ACETYLATION 1 DAY	REGENERATED CHITIN + HCl/ AcOAc 1 HOUR	SIGMA PURE CHITIN + HCl/ AcOAc 10 MINUTES
FTIR a)@	69%	79%	73%	71%	N.A.
FTIR b)#	52%	74%	>100	>100	N.A.
FTIR c)&	51%	74%	>100	>100	N.A.
SALICYL-ALDEHYDE*	96%	N.A.	98%	84%	78%

@ Domszy and Roberts (1985); # Shimahara and Takiguchi (1988); & Sannan *et al.* (1978); * Neugebauer (1989).

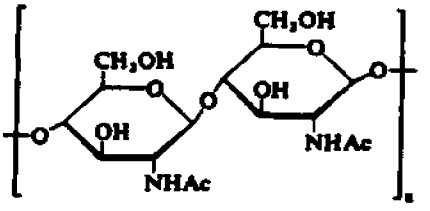
Appendix A.5

Saccharides for induction studies

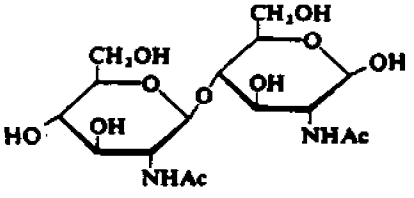
CELLOBIOSE



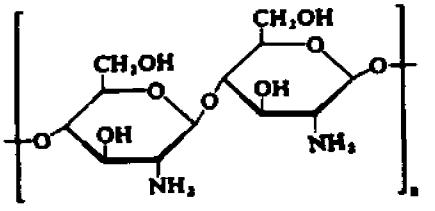
CHITIN



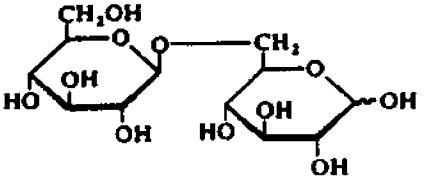
DI-N-ACETYLCHITOBIOSE



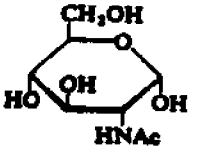
CHITOSAN



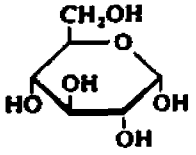
GENTIOBIOSE



N-ACETYLGLUCOSAMINE

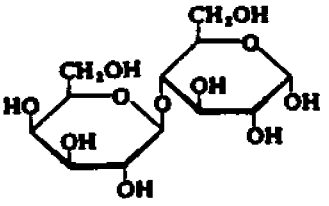


GLUCOSE

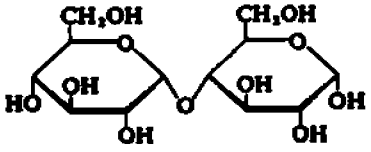


α-D-Pyranose-*form*

LACTOSE

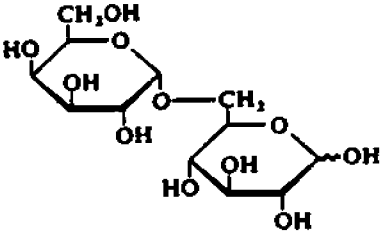


MALTOSE

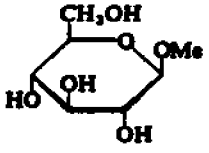


α-form

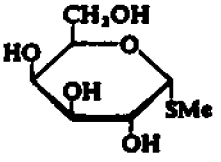
MELIBIOSE



METHYL-ß-GLUCOSIDE

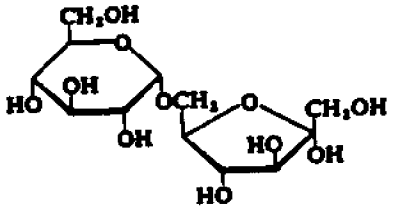


METHYL-THIOGALACTOSIDE

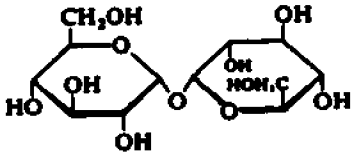


α-D-pyranose *form*

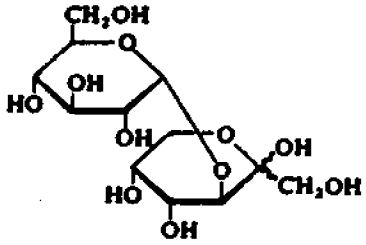
PALATINOSE



TREHALOSE



TURANOSE



Vita

Otto S. Gildemeister, son of Otto Gildemeister Peters and Rita Silva Vargas, was born in Concepcion, CHILE, on December 21, 1948. He attended the German School in Concepcion, Vina del Mar and Valparaiso. He attended Universidad Tecnica Federico Santa Maria in Valparaiso, from which he recieved a degree in Chemical Civil Engineering in 1982, while having a full-time position in the Department of Physics at the same University.

He married to Tatjana Abovich in 1983. They were blessed with two children, Karoline in 1985, and Reinhold, in 1987.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Otto S. Gildemeister

Major Field: Biochemistry

Title of Dissertation: STUDY ON TWO CHITIN-BINDING PROTEINS FROM VIBRIO PARAHAEMOLYTICUS

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

November 6, 1991